THE UNIVERSITY OF ALBERTA

"FACTORS INFLUENCING AXONAL REGENERATION IN THE MAMMALIAN SPINAL CORD"

A Dissertation
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of Master of Science (Surgery)

- Department of Surgery -
by
William John O'Callaghan, M.D.

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SYNOPSIS

In Chapter I, the literature concerned with previous investigations of regeneration in the mammalian spinal cord is reviewed. The purposes of the present experiment, and the hypothesis upon which the study is based, are stated.

Chapter II gives an account of the methodology employed in this project. Emphasis is placed on a method for ensuring complete transection of the rat spinal cord. The effects of chemotherapy and organotherapy on the sequence of events following spinal cord transection are tested.

In Chapter III, the results of the experiment are presented. Spinal axon regeneration ceased spontaneously in control rats. Reduction of scarring at the transection site did not lengthen the period of neurofibrillar growth. The regeneration of transected spinal cord axons was prolonged by the administration of a homogenate of central nervous tissue. Coordinated walking ability returned in 6 rats which had received homogenate injections. Axons were demonstrated histologically in the scar at the spinal cord transection site as late as 25 weeks postoperatively. Evidence that the functional return was due to spinal axon regeneration is presented.

In Chapter IV, the results of the experiment are discussed. An explanation is suggested for the beneficial effects of homogenate therapy on regeneration in the rat spinal cord.

The conclusions from this investigation are stated in Chapter V.

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CHAPTER I

I N T R O D U C T I O N

The sequelae of injury to the human vertebral column have been observed and recorded since ancient times. Most writers emphasized the almost invariable sequence of irreversible paraplegia, gradual debilitation and eventual death which accompanied extensive lesions of the spinal cord. It is only during the past hundred years that concerted attempts have been made to produce experimental lesions of the mammalian spinal cord in order that, by analogy, there may be a better understanding of the poor response of man to spinal cord transection.

Investigations of the events following spinal cord transection in mammals may be divided into two periods. Observations before 1930 were concerned mainly with the functional, gross anatomical and histological post-transection phenomena *per se*, with few attempts to alter the sequence of events. The early work helped to establish the concept of "abortive regeneration" of the spinal cord. Studies since 1930 have also dealt with the post-transection phenomena, but in addition various methods have been employed in an effort to alter the events so as to promote regeneration of the severed spinal cord axons.

Prior to the development of silver stains for axons and neurofibrils, investigators could find little evidence of regenerative activity at the site of spinal cord transection. Nerve fibers were seen in the scar as late as 6 months postoperatively in dogs (Dentan. 1873*)

and as early as 45 days in rabbits (Stroebe. 1894*), but agreement was general that these fibers arose from the spinal ganglia (Eichhorst and Naunyn. 1874*). Any functional return was believed to be on a reflex basis (Schiefferdecker. 1876*) and the concensus was that only spinal cord connective tissue and neuroglia regenerate (Enderlen. 1895*).

With the discovery and use of more specific staining methods by Ramon y Cajal and his contemporaries (circa 1905), nerve fibers could be definitely identified in the early scar at the site of transection or hemisection of the mammalian spinal cord. The morphological characteristics of these nerve fibers indicated that they were the result of a regenerative process, and not the products of degeneration of the cut axons. In the rabbit spinal cord, rings and loops at the end of axons were seen as early as the 4th day (Miyake. 1908*) and in the cat, signs of nerve regeneration were present at 20 days (Bielschowsky. 1909*). Ramon y Cajal (1906**) also transected cat spinal cords and found numerous fibers with terminal knob-like enlargements at 20 days. However, the number of new fibers gradually diminished after 36 days. The resorption of new fibers was noted

* Cited by Lee (1929)
** Cited by Ramon y Cajal (1928)

at 2 months in the rabbit spinal cord following partial section (Ssmarin. 1926*) and after 7 months, also in the rabbit, no nerve fibers could be found in the scar (Fickler. 1905*).

A proportion of the regenerating nerve processes in the post-transection scar was conceded to arise from the spinal ganglia. Fibers from the posterior roots grew towards the spinal cord in dogs (Marinesco and Minea. 1906*) and bifurcated on reaching the scar in kittens (D' Abundo. 1911*). Nevertheless, the majority of regenerating neurofibrils in the scar appeared to arise directly from severed central axons in the cord.

More recent histological investigations of the events following transection of the mammalian spinal cord have tended to confirm the earlier findings. Minor variations in the time of appearance of the first regenerating neurofibrils have been noted among the species examined, and among individual animals in the same species. The general sequence of events is, however, remarkably similar in all mammals studied. During the first week after transection, increasing numbers of neuroglial cells tend to separate the cord ends from the organizing blood clot at the operative site. Connective tissue ingrowth from the surrounding tissues begins early, and by 7 days there is an abundance of collagen between the cut cord

* Cited by Lee (1929)

ends in both the rat (Barnard and Carpenter. 1950) and the cat (Clemente. 1955). The sprouts of regenerating axons do not appear in the cord parenchyma until 7 days (rat) to 15 days (cat), and do not reach the transection site until 10 days (rat) to 30 days (cat) postoperatively. The scar by this time is cellular, transverse and relatively avascular. The regenerating neurofibrils may fail to penetrate the scar, in which case they atrophy (Clemente. 1955); they may grow along the transverse collagen fibers; or they may become recurrent in their course (Brown and McCouch. 1947). No regenerating neurofibrils were seen to cross the transection site in rats (Barnard and Carpenter. 1950), cats (Clemente. 1955) or dogs (Brown and McCouch. 1947).

Regenerative attempts by spinal cord axons have been observed in man. The process, although incompletely studied, resembles that seen in the lower mammals. Ringlets, bulbous enlargements, cones of growth and dividing fibers have been seen 23 days (Marinesco and Minea. 1910*) and 29 days (Perrero. 1909*) following traumatic transection of the spinal cord. More recently, Druckman and Mair (1953) found nerve fibers in and around the injured spinal cord in cases of syringomyelia, protruded intervertebral discs and myeloma of the spinal column. These aberrant fibers were similar to those seen in experimental

* Cited by Lockhart (1955)

lesions and appeared to arise from the long tracts, the decussating fibers of the anterior commisure, the anterior horn cells and the posterior roots. None of the newly regenerated fibers was observed to establish neuronal connections.

The observed failure of the adult mammalian spinal cord to regenerate aroused considerable speculation. It was postulated that conditions more favorable to regeneration might be present in the mammalian fetus than occur in the adult. This theory had as its basis the fact that restoration of morphological and functional continuity after spinal cord transection occurs in fishes and amphibia, both young and adult. Regeneration is generally more complete in the young forms and is accomplished without appreciable scarring. Metaplasia of cord ependymal cells into neuroblastic or neurilemma-like cells takes place and these send out nerve fibers which bridge the transection site. A similar phenomenon had never been observed in adult mammals. Whether "ontogeny recapitulates phylogeny" and whether the mammalian fetus passes through a development stage during which spinal cord regeneration is possible was the subject of a number of investigations.

The effects of spinal cord transection have been studied in fetal and newborn rats (Gerard and Koppanyi. 1926), fetal rats (Hooker and Nicholas. 1927), and fetal guinea pigs (Hess. 1956). Recovery of locomotor ability,

sensation or both occurred in all of the fetal rats and some of the newborn rats in Gerard and Koppanyi's (1926) series. Later histological examination of the spinal cords of these animals revealed that the spinal cord was, in many instances, incompletely transected (Gerard and Grinker. 1931). There was no evidence of anatomical regeneration of the cut axons, and functional return was ascribed to the residual intact axons and to the extensive "physiologic reorganization" which occurs in the immature spinal cord. Complete failure of nervous regeneration in the fetal rat spinal cord was reported by Hooker and Nicholas (Hooker and Nicholas. 1927, 1930; Nicholas and Hooker. 1928) who noted severe necrosis and vascular disturbances at the transection site. Any return of function was considered to be due to reflexes. Hess (1956) transected the spinal cord of fetal guinea pigs and observed that no outgrowths occurred from the severed central axons. Neurons of very young fetuses disintegrated completely after axon transection; those of older fetuses showed severe chromatolysis. The neuroglial proliferation, which began at about 5 days postoperatively, was neither organized nor extensive. Hess (1956) concluded that the absence of spinal cord regeneration during mammalian ontology was due to an inherent inability of mammalian central nervous cells to dedifferentiate; this ability, present in fishes and amphibia, was entirely lost in the phylogenetic evolution of the mammals.

The failure of normal function to return after complete transection of the mammalian spinal cord, together with the gross anatomical observation of a dense mass of scar tissue between the cord ends and the histological evidence of the lack of neural continuity between the stumps led to the concept of "abortive regeneration" (de Quervain. 1908). The concept was stated emphatically by Lee (1929) who concluded that "regeneration does take place in the central nervous system, but the process is abortive, many of the fibers undergoing resorption, and the ones that remain establishing no functional connections". The investigations thus far outlined have tended to support this concept.

Not all investigators believed that spinal cord regeneration was "phylogenetically impossible" in mammals. Many reasons were postulated for the abrupt cessation of the regenerative process; these include:

1) There are no neurilemma (Schwann) cells to provide nutritive or guiding pathways such as are present in the peripheral nerves (Ramon y Cajal. 1928).
2) Disturbances in the vascular pattern render the transection site ischemic (Nicholas and Hooker. 1928).
3) The connective tissue scar forms a barrier which the regenerating nerve fibers cannot cross.
4) The neuroglial scar limits regeneration.

5) The debilitation and metabolic disturbances after spinal cord injury permit only feeble attempts at regeneration by the transected spinal axons (Ramon y Cajal. 1928; Freeman. 1952).

Much ingenuity has been displayed in the attempts to alter the "unfavorable milieu" of the spinal cord transection site.

The observation that transected peripheral nerves will regenerate under favorable local conditions (such as the restoration of continuity of the neurilemma and endoneurium) led several groups of investigators to study the possibility that the peripheral Schwann sheath liberates trophic or nutritive substances or provides a guiding pathway for regenerating neurofibrils. Sugar and Gerard (1940) transected the spinal cord of young rats, in some of which implants of peripheral nerve and muscle were placed between the cord ends. Partial recovery of walking ability was noted in 13 of the animals with implants. In a few rats there was evidence of return of sensation (proprioception) distal to the spinal cord lesion. Electrical stimulation of the cerebral peduncles in several of the rats resulted in movements of the hind limbs. Histologically neurofibrils were observed to have grown into the implants. In the walking animals, bundles of nerve fibers could be seen spanning the transection site. Sugar and Gerard found that the main hindrance to regeneration was the dense

transverse connective tissue and glial scarring which occurred between the cord stumps. They concluded that anatomical and physiological regeneration in the rat spinal cord is aided by an implant of degenerating sciatic nerve.

Similar methods have been employed in attempts to enhance the regenerative process in cats and dogs. Brown and McCouch (1947) introduced peripheral nerve grafts, degenerated nerve emulsion or fetal central nervous emulsion between the ends of the transected spinal cord. No return of function, except reflex, occurred in any of the animals. Histologically, dense connective tissue scarring isolated the cut ends of the spinal cord. This scarring was increased in animals in which nervous tissue had been placed between the cord stumps. Controls showed less scarring than the treated dogs and cats. No regenerating neurofibrils were seen to cross the transection site.

Negative results were reported by Barnard and Carpenter (1949, 1950) who introduced homo- and autografts of fresh sciatic nerve, degenerated sciatic nerve, and muscle in the gap between the ends of the transected spinal cord in rats. No functional return was found beyond that of the "spinal" animal. Microscopically, dense connective scarring was present. Only very feeble attempts at regeneration by the cut axons were observed. The few regenerating neurofibrils penetrated the grafts for only short distances.

None crossed the scar. These investigators criticized the transection method used by Sugar and Gerard (1940), claiming that the operative procedure did not assure complete severance of the spinal cord. They also considered that the presence of "bundles of nerve fibers" spanning the transection site was evidence of incomplete transection of the cord. The functional return seen by Sugar and Gerard was ascribed by Barnard and Carpenter (1950) to residual intact axons, and not to regeneration of the spinal cord.

Massive scarring and no evidence of regeneration were also noted in the rat spinal cord by Freeman (1952) after nerve grafts, pulpified cord tissue, arachnoid and pia mater were placed between the cord ends. Feigin, Geller and Wolf (1957) could find no evidence of regeneration in rats after spinal cord transection and peripheral nerve implantation.

The marked increase in scarring which occurred when grafts of nervous tissue were introduced between the ends of the transected spinal cord led numerous investigators to search for a "biologically inert" substance that could be used as a guiding pathway for the regenerating neurofibrils. A porous plastic filter sheath of HA Millipore has been inserted between the proximal and distal stumps by several groups of workers with almost uniform results (Campbell et al. 1957; Andrew et al. 1959; Campbell and

Windle. 1960; Thulin. 1960). Connective tissue and neuroglial scarring was reduced and neurofibrils were seen to have grown into the graft, but there was no return of function. The studies were conducted mainly in cats and monkeys.

The apparent failure of the various grafting procedures to aid regeneration of axons across the transection site directed attention toward methods of reducing scar tissue formation. The two major components of the post-transection scar are connective tissue and neuroglia. A third component, which gradually decreases in amount, is made up of degenerating nervous tissue of spinal cord origin. A fourth component, usually not prominent, comprises the new blood vessels growing into the transection site.

Most investigators have recognized that undue trauma to the spinal cord increases the degeneration of nervous tissue around the operative site. This degenerating tissue was thought to increase scar formation. However, reduction of cord manipulation at transection resulted in little apparent decrease in connective tissue scarring, although there was less cord debris at the transection site.

Measures designed specifically to reduce connective tissue formation between the ends of the transected mammalian spinal cord have been successful in decreasing the mesodermal

scar, but have had little appreciable effect on the regenerative process. Davidoff and Ransohoff (1948) covered the cord stumps in cats with gall bladder or a gelatin capsule to prevent mesodermal encroachment from the surrounding tissues into the operative site. Although connective tissue was reduced and there was an adequate blood supply in the region, no regenerative capacity was demonstrated. Similar results have been reported by Gokay and Freeman (1952) after cortisone treatment, Freeman et al (1960) after intrathecal trypsin, and by Turbes, Freeman and Gastineau (1960) following irradiation of the spinal cord lesions. The absence of functional or histological evidence of regeneration after reduction of the connective tissue scar was believed by many investigators to be due to the barrier to regeneration presented by the neuroglial scar (Clemente. 1955).

Attempts to decrease or eliminate the glial scar have centered about the use of a purified pyrogenic bacterial polysaccharide complex (Piromen Baxter). This substance was found to have biological effects similar to those of ACTH. In experiments to determine possible uses of the bacterial pyrogen, cat and dog spinal cords were transected at various levels by Windle and Chambers (1950). Histological studies of the operative site up to 2 months after transection revealed a new growth of neurons into or across the cut. Some connective tissue scarring was present, but there

appeared to be no glial barrier. Between the severed ends of the cord there was a loose tissue containing non-nervous cells of a low order of differentiation. No evidence of functional regeneration was obtained.

Clemente et al (1951) treated spinal cats with Piromen and noted that there was reduction of both collagenous and glial scar tissue. Nerve fibers were seen to grow into a loose matrix of reticular and macrophage cells. Scott and Clemente (1951) were able to record action potentials in the spinal cord of Piromen-treated cats after stimulation above the lesion. The potentials could be detected 20 millimeters below the transection, and these investigators concluded that approximately 15% of the normal number of fibers in the cord had regenerated. Windle and Chambers (1951) reported that numerous blood vessels were present at the scar-cord junction in Piromen-treated cats and dogs, and that many macrophages occupied spaces between strands of non-nerve cells. Gokay and Freeman (1952) found that 16 of 33 rats recovered "useful function" after spinal cord transection and the administration of 1 microgram of pyrogen per day for 30 days. However, 15 of 66 control animals also developed "useful function". Littrell (1955) injected varying doses of Piromen into spinal cats and observed the development of coordinated walking in some of the animals. The early response was lost during long-term observation

(over 12 months). Associated with the functional regression was a constriction of the soft, regenerating cord parenchyma by a fibrous connective tissue scar.

Pyrogen therapy has not been shown by all investigators to benefit the regenerative process in the mammalian spinal cord. In a clinical study of patients with various neurologic disorders, Bailey, Rooke and Rodin (1952) could find no improvement in functional performance after Piromen administration. Lance (1954) found no evidence of either axon regeneration or glial scar reduction in Piromen-treated cats after unilateral section of the pyramidal tract in the medulla. No functional recovery was noted in cats (Arteta. 1956) or monkeys (Windle et al 1956) following spinal cord transection and pyrogen treatment.

Amid the numerous reports of "abortive regeneration" of the mammalian spinal cord and the failure of return of function despite many and varied methods of treatment, the results of studies by Freeman and his co-workers are somewhat exceptional. Freeman, Finneran and Schlegal (1949) reported spontaneous regeneration of axons in the spinal cord of rats. No definitive treatment was attempted. In 1952 Freeman reported spontaneous regeneration in the spinal cord of rats, cats and dogs with considerable return of function. Later information supplied by Freeman in review articles (1955, 1962) provides more detail about the earlier

studies. The first walking rat was noted in a series of animals made paraplegic for studies of calcium metabolism after spinal cord injury. In early experimental groups in which the regenerative potential of the spinal cord was specifically under observation, the cord was cut with a blade or scissors after having been delivered clear of its bony surroundings by means of a curved blunt hook. No functional return was seen in the first 175 animals. In later experimental groups, a sharp blade was passed circumferentially about the spinal canal, keeping the blade tip in continuous contact with bone. A total of 44 out of 1750 animals operated recovered walking ability closely approximating normal. Regeneration was considered to have occurred in the spinal cord. The reasons presented for the successful recovery of function were the excellent postoperative care given the animals, the minimization of catabolic changes, and the prolonged survival time.

Several objections have been raised to Freeman's claim of spontaneous regeneration of the mammalian spinal cord. The high incidence of urinary tract infection in the animals may have led to the production of pyrogens, by the infecting organisms, with beneficial effects on the regenerative process (Windle. 1955). Since newborn and young dogs and cats were used, restoration of morphology and function may not have been through axonal regeneration but by the normal developmental

growth of uninjured axons through the wound site (Chambers. 1955). Reflex walking after spinal conditioning has been observed in dogs after spinal cord transection (Shurrager and Culler. 1940). The method employed for spinal cord transection in the rats was similar to that used by Sugar and Gerard (1940). There is doubt whether this method ensures complete severence of the cord (Barnard and Carpenter. 1949). The results of Freeman's studies were not fully controlled histologically. In the dogs, the transection method employed by Turbes, Freeman and Gastineau (1960) resulted in residual intact ventrally-placed spinal cord fibers.

The generally-accepted criteria for the demonstration of regeneration in the transected mammalian spinal cord are:

1) Proof that the spinal cord was severed completely.
2) Clinical signs of restitution of motor and/or sensory function.
3) Evidence of nervous conduction across the transection site following electrical stimulation above or below.
4) Demonstration that the observed motor activity is not solely on a spinal reflex basis.
5) Re-establishment of histological continuity of axons across the transection site.

The results of attempts since 1930 to alter the process of "abortive regeneration" in the mammalian spinal cord either have been negative, or must be considered inconclusive because

of failure to fulfil all of the necessary criteria for unequivocal demonstration of axonal regeneration.

The present study was commenced under the hypothesis that regeneration of the mammalian spinal cord is not inevitably an abortive process, but may result in restoration of morphological and functional continuity provided that conditions conducive to regeneration are encouraged and that factors adversely affecting the regenerative process are minimized.

The purposes of the investigation have been to determine:

1) The extent of reflex activity in the spinal rat caudad to the transection site.
2) The histological sequence of events following spinal cord transection in the untreated adult rat.
3) Some of the effects of 6-methyl-delta-1-hydrocortisone* administration in normal and operated rats.
4) Some of the effects of Pseudomonas polysaccharide complex** administration in normal and operated rats.
5) The effect of administration of central nervous tissue homogenate derived from newborn rats on the regenerative process in the spinal cord of the adult rat.
6) The effect of combinations of methods of treatment where these methods have each been shown to alter a

* Depo-Medrol (Upjohn)
** Piromen (Baxter)

specific phase in the sequence of events following spinal cord transection.

The ultimate purpose of the investigation has been the development of a therapeutic regimen for the regular demonstration of regeneration of transected axons in the mammalian spinal cord, with permanent recovery of function.

CHAPTER II

M E T H O D O L O G Y

Healthy albino rats of the Sprague-Dawley strain were used. Initial studies involved male and female rats with a weight range of 95-215 Gm. Procedures were standardized to the 140-160 Gm female. A total of 700 rats was employed in the investigation, excluding the large number of newborn rats used as a source of central nervous tissue.

The animals were divided into four major groups. Rats not assigned to a series were those which died at operation (15) and those kept as normal weight gain controls (10).

I - OPERATIVE CONTROLS

a) Laminectomy only (5).

b) Cord transection, historical method (5).

c) Cord transection, present method (75).

d) Segmental cord resection (50).

II - METHYL PREDNISOLONE (DEPO-MEDROL)

a) Unoperated controls (50).

b) Steroid plus skin incision (40).

c) Steroid plus cord transection (185).

III - PIROMEN

a) Unoperated controls (10).

b) Piromen plus cord transection (75).

IV - CENTRAL NERVOUS HOMOGENATE

a) Transection plus homogenate (75).

b) Transection plus homogenate and Piromen (30).

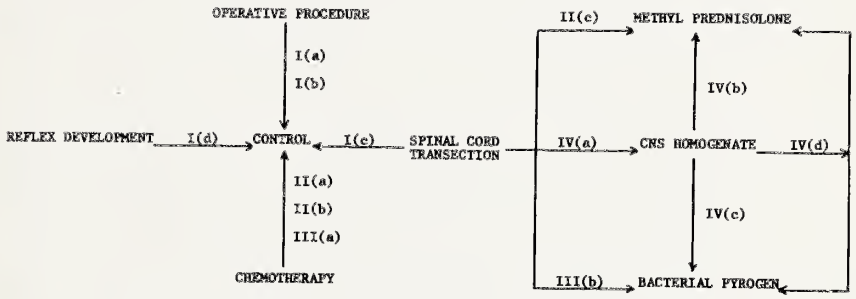

FIG. 1 - PLAN USED FOR THE ASSESSMENT OF POSSIBLE SPINAL CORD REGENERATION IN THE RAT WITH AND WITHOUT TREATMENT.

c) Transection plus homogenate and methyl prednisolone (45).

d) Transection plus homogenate, Piromen and methyl prednisolone (30).

A. OPERATIVE PROCEDURES

Under continuous ether anesthesia, the animals were placed in the prone position and the skin over the dorsal region shaved with fine electric clippers. The skin was then swabbed with a 90% isopropyl alcohol-iodine solution. Clean but not aseptic operative technique was used. Through a 3 cm. midline incision in the mid-dorsal region, the dorsal fat pad was exposed. The fatty tissue was elevated with thumb forceps and cut with iridectomy scissors. Any bleeding encountered was controlled by firm pressure with a cotton swab. The paravertebral muscles in the dorsal region were separated from the spinous processes by blunt dissection. Residual muscle bands were incised. Laminectomy of two or three dorsal vertebrae (usually D6, 7, and 8) was performed, with the spinal cord being exposed as widely as possible. A curved, blunt probe was then passed extradurally beneath and around the cord. The probe was designed to fit the inner contour of the rat vertebral canal following wide laminectomy (FIG.2), (FIG.3). Traction on the cord was avoided. The spinal cord was then cut with either a #11 Bard-Parker scalpel blade or iridectomy scissors. The bleeding encountered with this procedure usually ceased spontaneously. Excess blood at

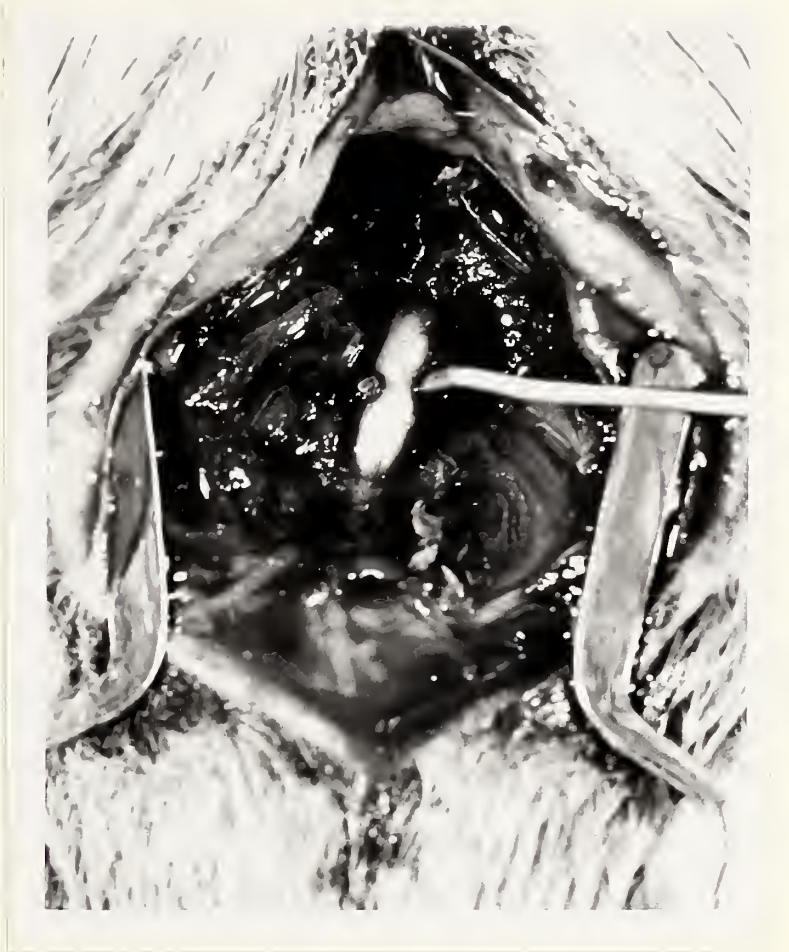

FIG. 2 - CURVED PROBE IN POSITION AROUND SPINAL CORD FOLLOWING WIDE MID-DORSAL LAMINECTOMY.

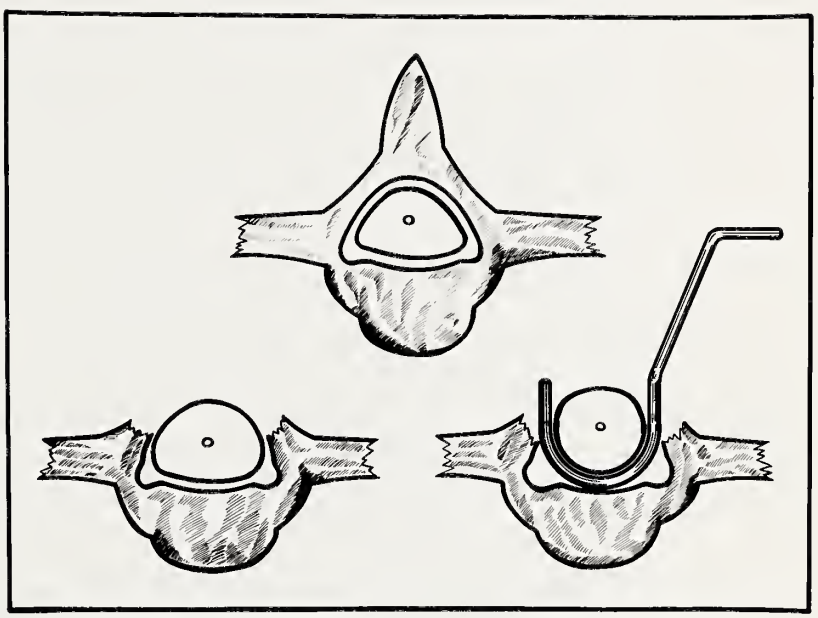

FIG. 3 - DORSAL VERTEBRA BEFORE LAMINECTOMY, AFTER LAMINECTOMY, AND WITH CURVED PROBE IN POSITION AROUND THE SPINAL CORD.

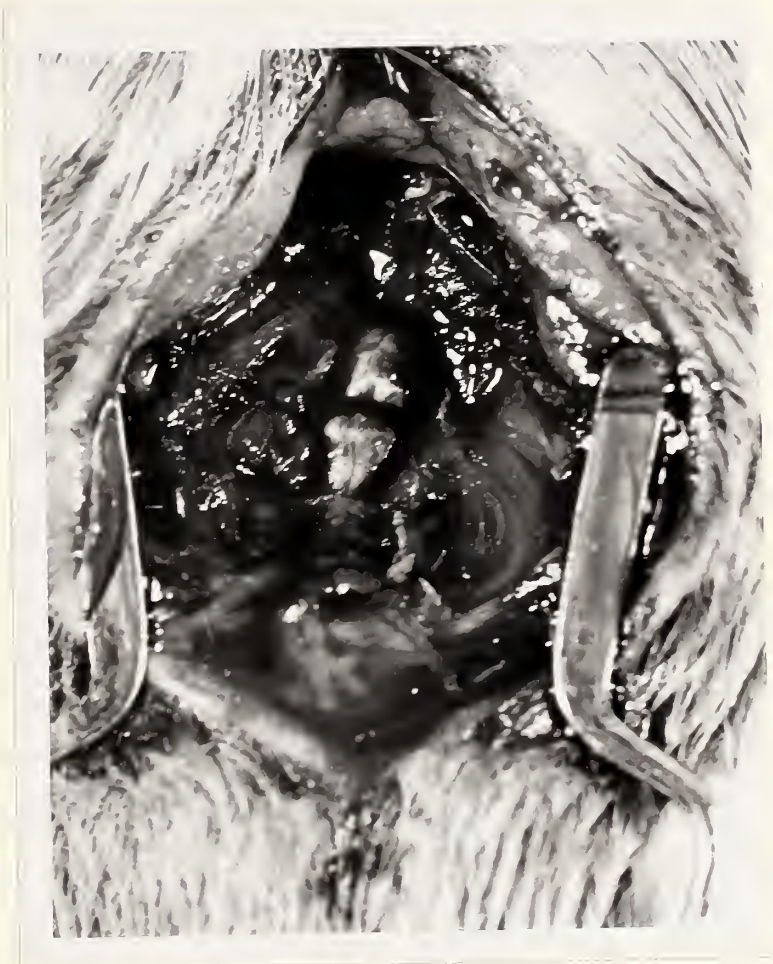

FIG. 4 - SEPARATION OF STUMPS OF SPINAL CORD FOLLOWING COMPLETE TRANSECTION.

the transection site was allowed to soak into a cotton swab. Pressure on the cord was avoided. Completeness of the transection was assured by unimpeded elevation of the curved probe between the cut ends of the cord. When a portion of intact cord was demonstrated by this method, the residual fibers were cut. Traction on the rat's tail resulted in separation of the cord ends to a distance of 1-2 mm. (FIG.4). The dorsal muscles and fat pad were then approximated with 5-0 chromic catgut, and the skin was closed with Michel clips. One or both ears were notched in accordance with a series numbering system, and the animal was placed in an assigned cage to awaken.

All rats in Groups II, III and IV, and those in Group I(c) which were subjected to spinal cord transection were operated upon by the above procedure. The animals in Group I(d) were prepared by this method except that a 3-5 mm. segment of spinal cord was excised. The effect of laminectomy alone, without spinal cord transection, was noted in Group I(a). Cutting the cord without prior placement of an extradural blunt hook, probe or seeker was investigated in Group I(b). The method was considered inadequate to assure complete transection of the spinal cord.

B. POSTOPERATIVE CARE

The hindquarters of the paraplegic rats were dipped in a saturated solution of picric acid in an effort to deter cannibalism of the insentient feet and legs. The animals were then placed in wire mesh - floored cages. Fresh water

and commercial rat diet* were provided daily. The urinary bladder was expressed 3 times daily until bladder control became regularized, and thereafter once daily for a month. Soiled animals were bathed. Periodic examination for acute urinary retention was made in the later postoperative months. Bowel care did not present a problem as fecal pellets were dropped by the first postoperative day. Due to the lack of isolation facilities, rats suspected of developing pneumonia in the postoperative period were sacrificed.

C. INVESTIGATION OF EFFECTS OF CHEMOTHERAPY AND ORGANOTHERAPY

The operative control rats in Group I received only routine postoperative care.

In Group II, methyl prednisolone was administered to normal and operated rats and some of the effects of this drug noted. Animals in Group II(a) were divided into 5 experimental series of 10 rats each. The various series received 8, 1, 0.6, 0.4 and 0.2 mg. of methyl prednisolone respectively. Weight change, development of infection and the apparent duration of drug action were noted at the different dosages. A single injection of the drug was given into the dorsal muscle mass. The effect of methyl prednisolone, also in a single intramuscular injection, on the duration of the healing process in a skin incision was noted in Group II(b). Under continuous ether anesthesia,

*Purina Labena (Ralston Purina Company)

the skin in the midline dorsal region was incised. Four series, of 10 animals each, received 8, 1, 0.6 and 0.4 mg. of methyl prednisolone respectively directly into the dorsal muscle mass. The observation period for all the above rats was 3 weeks. The animals were weighed daily. In Group II(b) the skin clips were removed from an animal in each series at 2 day intervals and separation of the wound edges observed and recorded.

The effect of methyl prednisolone on the formation of scar tissue at the site of spinal cord transection was studied in Group II(c). The spinal cord was cut by the described method and the first injection of the drug given into the dorsal muscle mass at operation. Three dosage schedules were employed. In a first series of 80 rats, 8 mg. of methyl prednisolone was administered at operation and every two weeks thereafter. In a second series of 30 rats, 2 mg. was given at operation and every two weeks thereafter. In the third series, 75 rats received 0.2 mg. at operation and at 14 days postoperatively, and 1 mg. every two weeks thereafter. Daily weight was recorded in the latter series for the first 2 weeks after operation.

Possible effects of purified bacterial pyrogen (Piromen) in normal and paraplegic rats were investigated in Group III. The Piromen was stored at a temperature of 4-9 degrees Centigrade before administration, and was used

before the expiry date. Three lots of the drug, with different expiry dates, were tested for biological activity or evidence of deterioration in storage. Since the rat is not recommended as a biological test animal for febrile response to pyrogens, the effect on temperature of a 10 ml. per Kg. injection of Piromen was noted in the rabbit. Chemical and color tests were also used - the ferric chloride-potassium ferricyanide reaction and the permanganate test (Carter. 1930) for oxidizable substances. Ultraviolet absorption at a wave length of 2650 A was examined. Finally, 10 normal rats (Group III(a)) were given 1 microgram of Piromen per day, injected intraperitoneally, and the daily weight compared with a group of 10 rats kept as normal weight gain controls.

In Group III(b) the spinal cord was transected in 75 rats. Each received a daily intraperitoneal injection of 1 microgram of Piromen, beginning on the first postoperative day and continued for 5 weeks. The urinary bladder was expressed manually and emptied as completely as possible before each intraperitoneal injection. Otherwise, standard postoperative care was given.

Accuracy of drug dosage administration was attempted in Groups II and III by the use of a finely-calibrated 1 ml. insulin syringe and a #25 gauge needle. The methyl prednisolone was mixed into suspension immediately before use. The Piromen was taken out of cold storage immediately prior to injection.

In Group IV possible effects of the administration of central nervous tissue homogenate alone and in combination with chemotherapy in paraplegic rats were investigated.

The homogenate was prepared immediately prior to use. Three hour- to three-day-old newborn rats were lightly anesthetized with ether. The vertebral canal and cranium were opened dorsally in the midline with a #11 scalpel blade or iridectomy scissors. The brain and spinal cord were then removed with fine forceps, placed in a SGA C-9290 homogenizer and covered with distilled water. The procedure was repeated until sufficient central nervous tissue had been collected (i.e. n/5 ml., where n=number of rats to be injected). The tissue was then homogenized manually until a translucent suspension was obtained. The suspension was taken up into a syringe through a 21 gauge needle and distilled water added to make a total volume of n ml. The contents of the syringe were mixed thoroughly before injection.

All rats in Group IV were subjected to spinal cord transection by the described method. An intraperitoneal injection of homogenate was given on the second postoperative day and every second day thereafter for 4 weeks. The volume of homogenate administered each injection was 1 ml., consisting of 1 part nervous tissue in 4 parts distilled water.

In Group IV(a) 75 paraplegic rats received intraperitoneal injections of homogenate and no chemotherapy.

In Group IV(b) 30 paraplegic rats received injections of homogenate. In addition, 1 microgram of Piromen was administered intraperitoneally on each of the first 28 days postoperatively, and for 5 consecutive days per week thereafter.

In Group IV(c) 45 paraplegic rats received homogenate. In addition, 30 of these rats received 8 mg. methyl prednisolone intramuscularly at operation and every two weeks thereafter. The remaining 15 rats received 2 mg. methyl prednisolone intramuscularly at operation and every two weeks thereafter.

In Group IV(d) 30 paraplegic rats received homogenate. In addition, 1 microgram of Piromen was administered intraperitoneally on each of the first 28 days postoperatively; and 1 mg. methyl prednisolone was injected intramuscularly on the 28th day and every two weeks thereafter.

D. FUNCTIONAL EVALUATION

Normal rats were studied prior to surgery to determine typical locomotor activity and response to noxious stimuli. The rats were observed while walking on smooth and rough surfaces, and while running, climbing and jumping. The responses to pinching of the foot or tail, sham dropping, sudden lifting and light touch were noted and recorded. Rats were observed while climbing to determine typical placing and seeking movements of the hind limbs. The response of normal rats to irritating materials placed on the snout was noted. Comparisons were made of the activity of healthy rats and rats with upper and lower respiratory infections.

Rats with tumors, edema of the feet or hind limbs, missing digits, and any type of infection were excluded from surgery.

In all rats with spinal cord transection or segmental resection, the time of appearance, type and extent of movement distal to the operative site in response to various stimuli were observed and recorded. The rats were examined daily for alterations in reflex activity until the 21st postoperative day; thereafter formal testing of reflexes was done every second week. Tests for evidence of sensation distal to the operative site were conducted bi-weekly. Daily observation for spontaneous movement of the hind limbs was maintained for the duration of the experiment. Records were kept of the functional performance of each rat.

E. ELECTRICAL STIMULATION OF BRAIN STEM AND SPINAL CORD

Electrical stimulation of the brain stem and spinal cord was performed at the end of the desired observation period for rats from each group. Two surgical approaches to the central nervous system were used.

Under continuous anesthesia, the rats were placed in the prone position. The skin over the dorsum of the head, neck and thorax was shaved. In rats weighing less than 250 Gm. a midline scalp incision was made and carried down to the periostium. The temporal muscles were incised and retracted laterally. A rongeur was used to remove a 0.5-1.0 sq. cm.

area of bone from the dorsum of the skull. The bleeding encountered was controlled with pressure (for soft tissues) and tamponade with small pieces of muscle (for bleeding bone). Steel electrodes were inserted obliquely into the foramen magnum and withdrawn slightly. In rats weighing more than 250 Gm. a midline incision was made in the cervical region. The dorsal muscles in the neck were dissected from the midline and retracted laterally. The rat's head was then flexed and the occipital prominence of the skull dissected clear of muscle. The posterior atlanto-occipital ligament was incised transversely in its full width. Steel electrodes were inserted initially craniad into the brain stem, and then caudad into the spinal cord. With either approach, the in situ electrodes were connected to an American Electronics #751 stimulating apparatus. The anesthesia was then lightened and the effects of electrical stimulation of the brain stem and spinal cord noted. The response of normal rats was compared with that of rats stimulated after spinal cord transection.

F. HISTOLOGICAL STUDIES

Two methods of formalin fixation of the spinal cord were compared. In the first method, the rat was given a lethal amount of ether. The dorsal portion of the vertebral column was excised en bloc, cleared of most of the attached muscle, placed in an assigned container, and covered with 10% formalin. The spinal cord was removed from the vertebral canal after two weeks.

In the second method, the rat was deeply anesthetized with continuous ether anesthesia. The thorax was opened parasternally. A #21 gauge needle attached to saline-filled polyethylene tubing was inserted into the left ventricle or the aorta. A Sigmamotor pump (Model TM11) was used to perfuse normal saline through the rat's circulatory system at a rate of 2.5 ml. per minute. When a relatively clear return was observed from the distal end of the transected inferior vena cava in the chest, perfusion of 10% formalin was begun. Formalin perfusion was continued for two minutes longer than the time previously noted as adequate for saline perfusion in that particular animal. The dorsal vertebral column was excised en bloc and refrigerated for one hour. The spinal cord was then removed from the vertebral canal and placed in 10% formalin for 2-4 days.

Following fixation, the spinal cord was sectioned at 6-10 microns. The staining methods employed were routine hematoxylin and eosin, Gomori's trichrome stain for connective tissue, Holzer's stain for glia fibers, Bodian's method for nerve fibers and nerve endings, and a modified Bodian's Protargol method (FIG. 5).

1. Wash tissues in distilled water.
2. Dehydrate in graded alcohols (50, 70, 95%, and absolute).
3. Clear in xylol.
4. Embed in paraffin.
5. Section 10 u.
6. Mount every third section serially.
7. Deparaffinize tissues.
8. Hydrate tissues to distilled water.
9. Mordant in 1.0% lead or cupric nitrate dissolved in Pearson's buffered water at pH 3.7 for 24 hours at 37 degrees C.
10. Rinse in distilled water for two minutes (two changes).
11. Place copper metal at the bottom of each slide dish containing the sections; pour distilled water on top of the sections and dust Protargol on top of the wster (0.5%. Protargol); allow it to stand until dissolved (approx. 35-40 min.); leave for 24 hours at 37 degrees C.
12. Rinse in distilled water for 1 minute (2 changes).
13. Reduce in Pearson's reducer at 55°C. until the tissues turn to a golden amber color. (Approx. 3 minutes).
14. Tone in 1% gold chloride.
15. Rinse in distilled water (2 changes).
16. Rinse in 1% oxalic acid for 2 minutes.
17. Rinse in distilled water (2 changes).
18. Dehydrate in alcohol series.
19. Clear in xylol.
20. Mount sections in Permount.

FIG. 5 - MODIFIED BODIAN'S PROTARGOL METHOD FOR DEMONSTRATING FINE MYELINATED AND UNMYELINATED NERVE FIBERS.

CHAPTER III

O B S E R V A T I O N S

Rats from each series were sacrificed at various intervals postoperatively for histological study of the spinal cord transection site. A representative number was maintained for long-term observation and functional evaluation. The maximum period of observation was 21 weeks. No histological study was possible of the spinal cord of rats which died spontaneously.

A. OPERATIVE CONTROLS

None of the 5 rats which underwent mid-dorsal laminectomy without spinal cord transection (Group I(a)) had a motor and sensory deficit of greater than 15 minutes duration postoperatively. Immediately after recovering from the anesthetic, the rats used only the forelimbs for locomotion; the inert hindquarters were dragged. There was no vocalization or struggling in response to pinching of the feet or tail. Within 5-15 minutes after operation, all of the rats made spontaneous movements of the hind limbs and normal walking ability returned. In 2 of the rats, the curved probe had been passed around the spinal cord. These rats did not differ functionally from those with laminectomy alone. Examination of the operative site after 1 month revealed a small amount of scar tissue connecting the dura and the overlying dorsal muscle mass in three of the rats. The spinal cord of one rat was examined histologically and showed no abnormality.

The 5 rats in Group I(b) underwent spinal cord transection without the prior placement of an extradural probe.

Three of the rats began to make spontaneous movements of one or both hind limbs in the second postoperative week, and one of these responded to painful stimuli below the level of operation. Walking ability increased rapidly in these 3 rats until approximately 21 days postoperatively, when it was practically indistinguishable from normal. The spinal cord was considered to be incompletely cut, with residual intact fibers accounting for the early functional return. The other 2 rats remained paraplegic. One died at 17 days from rupture of the urinary bladder; the second was maintained for 4 months and showed no evidence of functional return. The spinal cord transection method used in Group I(b) was considered inadequate and was not employed in any other series of rats.

In Group I(d) a study was made of the sequence of reflex development in the paraplegic rat. The 50 rats in this group underwent resection of a 3-5 mm. segment of mid-dorsal spinal cord. Movement of the hindquarters in response to various methods of stimulation was found to become more complex as the time postoperatively increased. A record was kept of the functional performance of each rat. Information obtained from this study was used to compile a "schedule" (FIG. 6) of reflex development which was employed as a standard of comparison with similar schedules for rats with transection of the spinal cord. Rats with respiratory or urinary tract infection were found to have retarded reflex development.

LEG FLEXION
- ipsilateral foot pinch (0)
- ipsilateral partial flexion of leg (3)
- ipsilateral abdominal pressure (3)
- contralateral leg traction (3)
- cool water on abdomen (3)
- sham dropping (6)

LEG EXTENSION
- tail pinch (1)
- contralateral foot pinch (3)
- contralateral leg traction (6)
- thoracic lift (6)
- hindquarter drag (6)

TAIL WITHDRAWAL
- tail pinch (3)
- thoracic lift (12)

RHYTHMIC STEPPING
- sudden release abdominal pressure (4)
- abdominal pressure (6)
- tail pinch (7)
- contralateral foot pinch (7)
- contralateral leg traction (10)
- cool water on abdomen (10)
- hindquarter drag (10)

TOE FANNING
- contralateral leg traction (10)
- contralateral foot pinch (11)
- thoracic lift (11)
- tail pinch (12)

SCRATCHING
- abdominal touch (12)

FIG. 6 - REFLEX RESPONSES OF RATS WITH SEGMENT OF SPINAL CORD EXCISED, TYPE OF STIMULATION TO ELICIT EACH REFLEX, AND EARLIEST DAY POSTOPERATIVELY REFLEX OBSERVED.

The distinct reflex responses noted were leg flexion, leg extension, tail withdrawal, rhythmic stepping, fanning of the toes and scratching. The flexion response consisted of a slow, steady flexion of the hip, knee, ankle and toes, with initial abduction at the hip followed by adduction. The extension response consisted of a rapid, jerking extension of the hip, knee and ankle with flexion of the toes. By approximately 14 days postoperatively, further progression in reflex development did not occur. The final state of the paraplegic rat consisted of rhythmic stepping movements of the hind limbs as the hindquarters were dragged over a rough surface during the rat's forward locomotion. This stepping was increased if the rat was made to climb an inclined screen. If the rat moved up the 45 degree incline rapidly, one or both hind limbs could occasionally be observed to make rapid, repetitive scratching movements. Coordinated movements of the front and hind limbs were never observed. The weight of the hindquarters was never supported on the feet. The toes were never used actively during forward locomotion. Apparent spontaneous movements of the hind limbs were occasionally seen, while the rat was at rest, but these were noted to coincide with defecation or the automatic expulsion of urine from the bladder. These movements were never observed before automatic control of the bladder had been achieved (2-3 weeks postoperatively).

None of the rats in Group I(d) showed evidence of recovery of sensation distal to the operative site during the

period of observation (up to 21 weeks). None showed locomotor ability resembling that in normal unoperated rats. No hind limb movements resulted from electrical stimulation of the brain stem and spinal cord in any of the 20 rats tested by this method. Gross anatomical observation of the operative site in 21 rats revealed two conditions to be present. In 14 rats the proximal and distal cord stumps were capped by scar tissue which adhered directly to the ventral portion of the vertebral canal. There was no direct continuity between the cord stumps. In 7 rats the cord ends were connected by a tissue bridge which was shown on microscopic examination to consist of dense connective tissue. Silver stains revealed neurofibrils in the scar of only one spinal cord. This cord had been taken from a rat sacrificed 4 weeks after operation. The neurofibrils were sparse, and could be found only in the scar immediately adjacent to the cord ends. None was seen in the mid-portion of the scar.

Group I(c) consisted of 75 rats in which the spinal cord was transected in the mid-dorsal region. The sequence of reflex development postoperatively was similar to that found in Group I(d). Functionally, no rat progressed beyond the paraplegic state and there was no return of sensation noted distal to the operative site. The hind limbs made rhythmic stepping movements as the hindquarters were dragged, but voluntary locomotion was entirely confined to the fore-limbs. There was no coordination between the front and hind

limbs, the weight of the hindquarters was not supported on the feet, and the toes were never used actively during forward locomotion.

In normal rats lightly anesthetized with ether, hind limb response to electrical stimulation of the brain stem and spinal cord occurred at strength 1 volt, frequency 5 pulses per second, and duration 1 millisecond. The hind limb response consisted of a fine tremor in time with the stimulation frequency. No hind limb movements resulted from electrical stimulation of the brain stem and spinal cord in any of the 23 rats in Group I(c) tested by this method. A wide range of stimulation strength (.01-10 volts), frequency (1-60 pulses per second) and duration (0.5-20 milliseconds) was tested with negative results.

In some rats in Group I(c) prolonged or profuse hemorrhage occurred from the operative site (cranium, vertebrae) prior to electrical stimulation. The hemorrhage was followed by respiratory arrest which was succeeded in turn by violent, clonic hind limb movements after 10-15 seconds. Cardiac arrest followed in another 30-45 seconds, and the hind limb movements ceased.

Violent kicking movements of the hind limbs were also observed in paraplegic rats during the perfusion of saline and formalin through the rat's circulatory system at the time of sacrifice. The movements would occasionally begin after approximately 15 ml. of saline had been perfused, but were

always observed shortly following the onset of formalin perfusion.

The results of the investigation outlined thus far have yielded several important considerations. Laminectomy, exclusive of spinal cord transection, caused a transient but reversible paraplegia, with full recovery of motor and sensory function occurring within 5-15 minutes postoperatively. In the second week postoperatively, rats with an incompletely transected spinal cord began to show return of motor and/or sensory function. Rats with a segment of spinal cord excised were functionally identical with rats in which the spinal cord had been transected by the present method. There was no return of sensation distal to the operative site. There was no return of normal locomotor ability. In rats with spinal cord transection, the rhythmic stepping movements of the hind limbs during forward locomotion were identical with those movements seen in rats with a segmental resection of spinal cord. The maximum period of observation was 21 weeks postoperatively. No movements of the hind limbs resulted from electrical stimulation of the brain stem and spinal cord in either the rats with spinal cord transection or those with segmental cord resection.

To supplement the functional and electrophysiological data, histological studies were conducted. Rats from Group I(c) were sacrificed at intervals postoperatively to determine the histological sequence of events at the site of spinal cord transection.

During the first 3 days postoperatively, the small gap between the cord ends was filled with friable blood clot loosely attached to the spinal cord stumps. Histological examination on the 4th postoperative day revealed signs of degeneration in the spinal cord. The nuclei of most neurons near the operative site were eccentrically-placed in the cell, while others were hyperchromatic. Immediately adjacent to the operative area, fragments of neural tissue and many macrophages were present.

By 7 days postoperatively (FIG. 7), organization of the blood clot had begun. Large capillaries and abundant collagenous tissue were present in the lateral aspects of the blood clot, while small capillary buds and thin transverse strands of connective tissue were observed in the central area. In several spinal cords a large, blood-filled cyst occupied the transection site. Connective tissue infiltration followed the borders of this cyst. Neuroglial tissue, several cells thick, covered the cut cord ends and separated the spinal cord from the organizing blood clot. Macrophages were present in large numbers. Silver stains revealed neurofibrils with bulbous ends in the spinal cord and the adjacent neuroglial scar. No neurofibrils were seen in any portion of the organizing blood clot.

By 14 days postoperatively (FIG. 8), large blood vessels were present in the central area of the organizing blood clot. Scattered strands of connective tissue traversed the width of the transection site. The cord-scar junction was

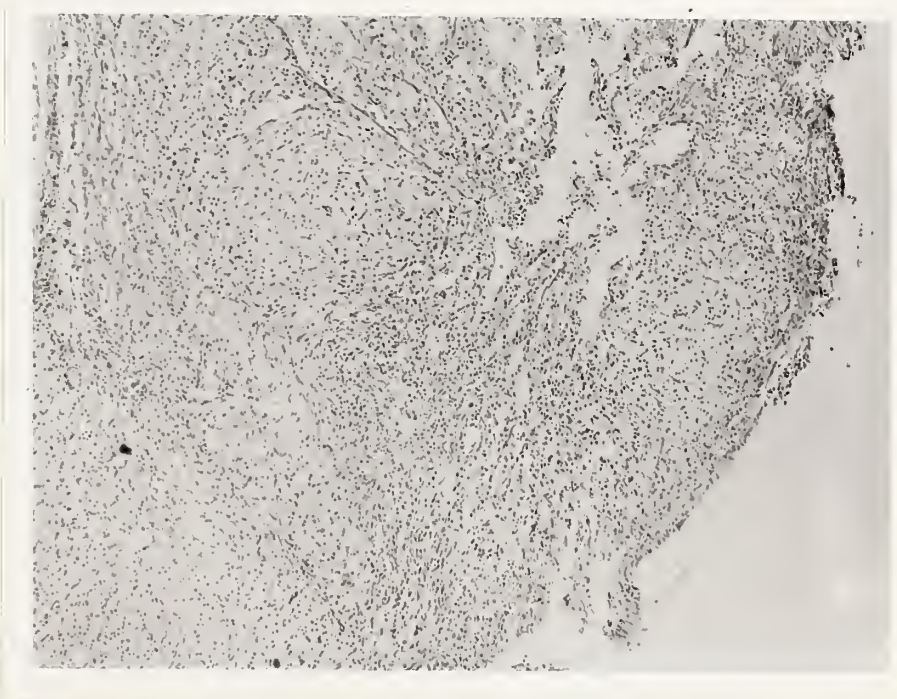

FIG. 7 - BEGINNING ORGANIZATION OF BLOOD CLOT AT SITE OF COMPLETE SPINAL CORD TRANSECTION (7DAYS). PROXIMAL CORD STUMP AT TOP OF PHOTOMICROGRAPH IN FIGS. 7-14. THICKNESS OF SECTION: 8 MICRONS. STAIN: HEMATOXYLIN AND EOSIN. MAGNIFICATION: X50.

poorly demarcated, and connective tissue from the organizing blood clot blended with the neuroglial scar capping the cord ends. Silver stains revealed regenerating neurofibrils in the neuroglial scar and in the most adjacent part of the connective tissue scar. The neurofibrils tended to grow along the connective tissue strands, but many were seen to have crossed short distances apparently without the guidance or support of a visibly solid structure. No neurofibrils were seen in the central area of the organizing blood clot.

By 3 weeks postoperatively (FIG. 9), the scar at the transection site was very cellular. Transverse connective tissue fibers were abundant in the central area. Regenerating neurofibrils were present in the relatively loose, cellular mesodermal-neuroglial area of the scar near the cord ends, but were absent from the central area of transverse fibrous collagenous tissue. Where a large, fluid-filled cyst was present between the cord ends, neurofibrils were not present in the connective tissue around the lateral aspects of the cyst.

By 4 weeks postoperatively (FIG. 10), the neuroglial scar had become approximately 30 cells thick. There was good demarcation at the transection site between the neuroglial component of the scar capping the ends of the transected spinal cord, and the central zone of transverse, cellular collagenous tissue. Neurofibrils were abundant in both proximal and distal

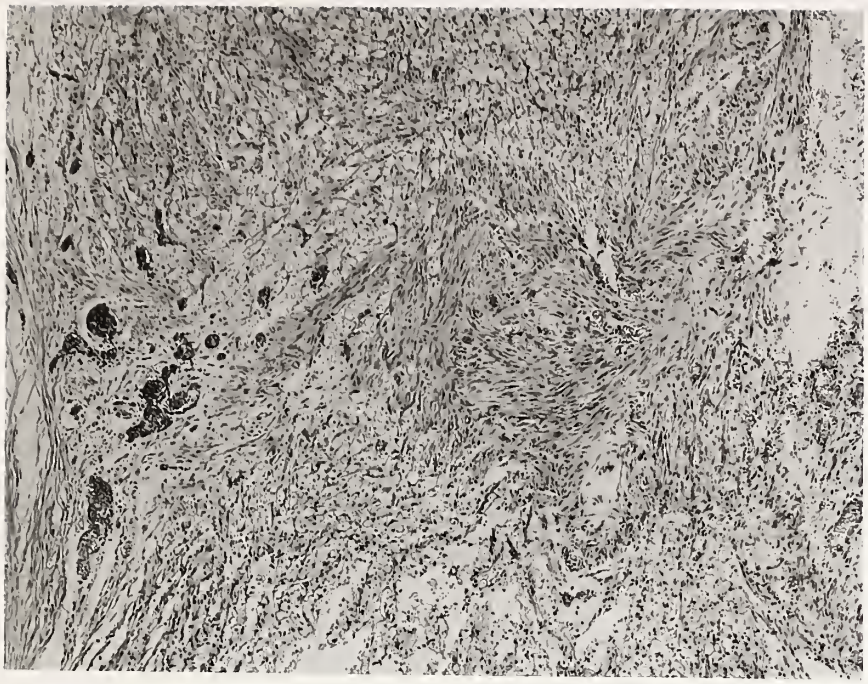

FIG. 8 - EARLY FORMATION OF TRANSVERSE CONNECTIVE TISSUE (14 DAYS) 8 MICRONS; HEMATOXYLIN AND EOSIN; X50.

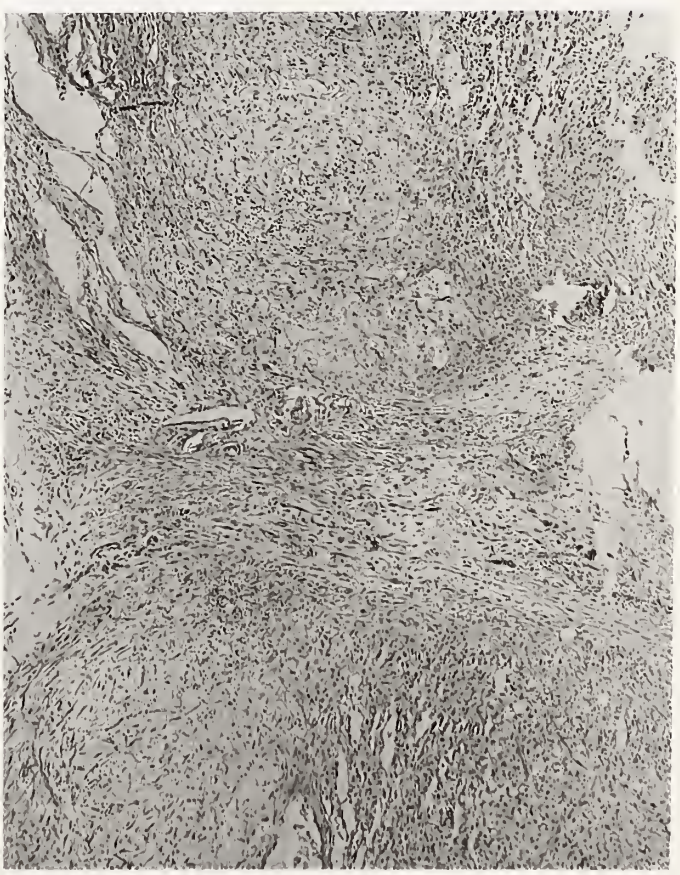

FIG. 9 - INCREASED CONNECTIVE TISSUE FORMATION AT TRANSECTION SITE (3 WEEKS); 8 MICRONS; HEMATOXYLIN AND EOSIN; X50.

neuroglial scars, but were very sparse in the connective tissue. The regenerating neurofibrils were longitudinally-oriented in the neuroglial tissue adjacent to the cut cord ends, but assumed a transverse position as the connective tissue scar was approached. The few neurofibrils in the connective tissue were in the proximo-lateral aspects of the scar, where the connective tissue fibers lay longitudinally before sweeping transversely through the mid-zone of the transection site.

By 5 weeks postoperatively (FIG.11), neuroglia and macrophages were less prominent than at 4 weeks. In several spinal cords a cyst-like structure had developed between one or both spinal cord stumps and the connective tissue scar. The appearance was one of separation of the transverse collagenous tissue from the spinal cord ends. This cystic structure differed from the hemorrhagic cysts seen in the early postoperative period. The "hemorrhagic cyst" was bounded both proximally and distally by transverse connective tissue. The "retraction cyst" was bounded by the spinal cord stump on one side, and by transverse connective tissue on the other. Strands of neuroglia were occasionally seen bridging this cystic area. Silver stains revealed neurofibrils in these neuroglial strands. Regenerating neurofibrils were not seen in any part of the connective tissue scar.

By 6 weeks postoperatively (FIG. 12), the connective

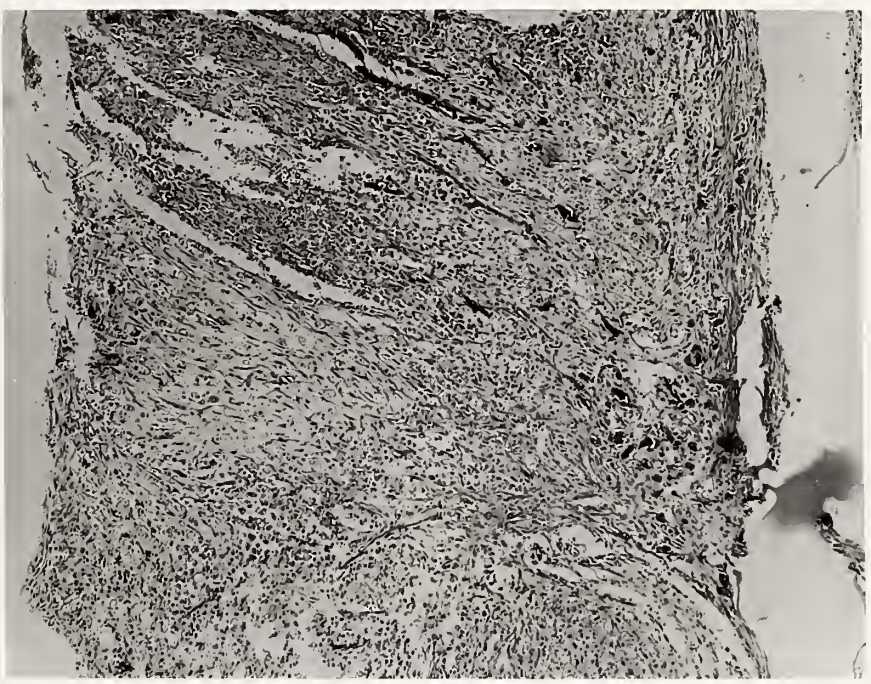

FIG. 10 - TRANSECTION SITE 4 WEEKS; 8 MICRONS;
HEMATOXYLIN AND EOSIN X50.

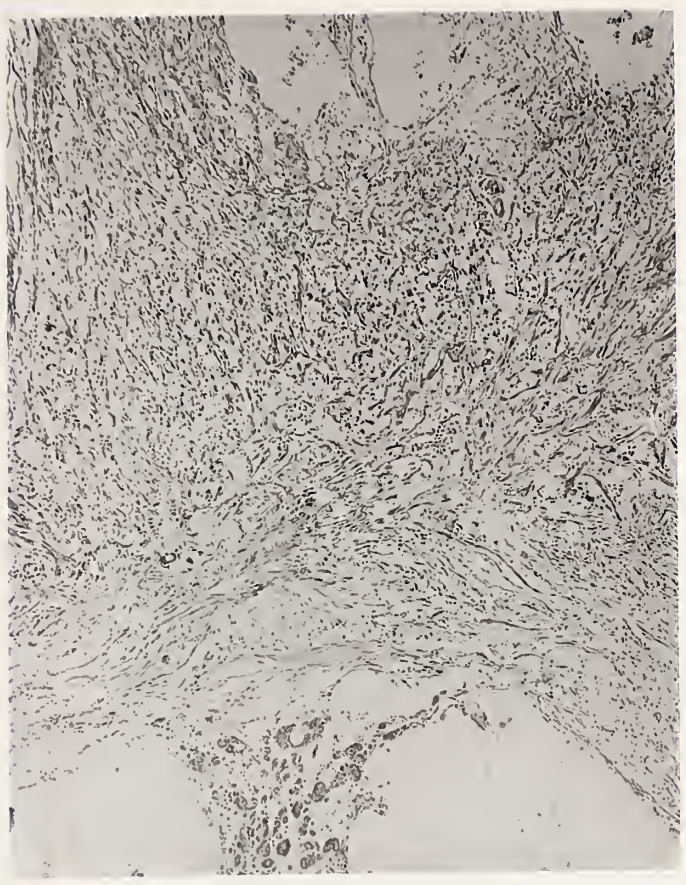

FIG. 11 - SEPARATION OF CORD STUMPS FROM SCAR (5 WEEKS); 6 MICRONS; HEMATOXYLIN AND EOSIN X50.

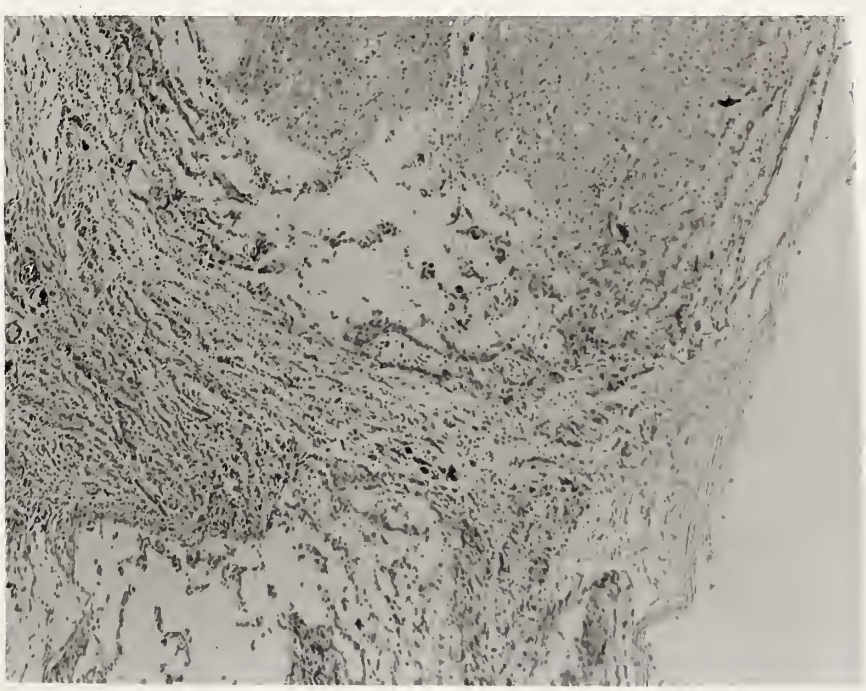

FIG. 12 - TRANSVERSE FIBROUS CONNECTIVE TISSUE AT TRANSECTION SITE (6WEEKS); 6 MICRONS; HEMATOXYLIN AND EOSIN X50.

tissue scar had become less cellular and more fibrous. The neuroglial scar consisted of a cap, 10-15 cells thick, closely applied to the ends of the transected spinal cord. Neurofibrils were very sparse in the neuroglial scar. An occasional neurofibril could be seen in the lateral aspect of the connective tissue scar. These nerve processes could be followed for only short distances in the connective tissue. No continuity was demonstrated between these neurofibrils and either the spinal cord stumps or the dorsal roots.

The appearance of the transection site at 9 weeks and at 12 weeks (FIG. 13) postoperatively differed little from that at 6 weeks. The mesodermal scar was more fibrous. Neurofibrils were not demonstrated in either the connective or neuroglial tissue. At 20 weeks postoperatively (FIG. 14), a dense, transverse, fibrous scar completely isolated the ends of the spinal cord. There was no evidence of central nervous axon regeneration.

In summary, neurofibrils with enlarged bulbous tips indicating axonal regeneration appeared in the spinal cord within the first week following complete spinal cord transection. Formation of a moderate amount of fibrous connective tissue preceded the appearance of regenerating neurofibrils at the transection site. The neurofibrils tended to grow out of the spinal cord stumps and along the loose strands of connective tissue. Early neurofibrillar growth was profuse through all

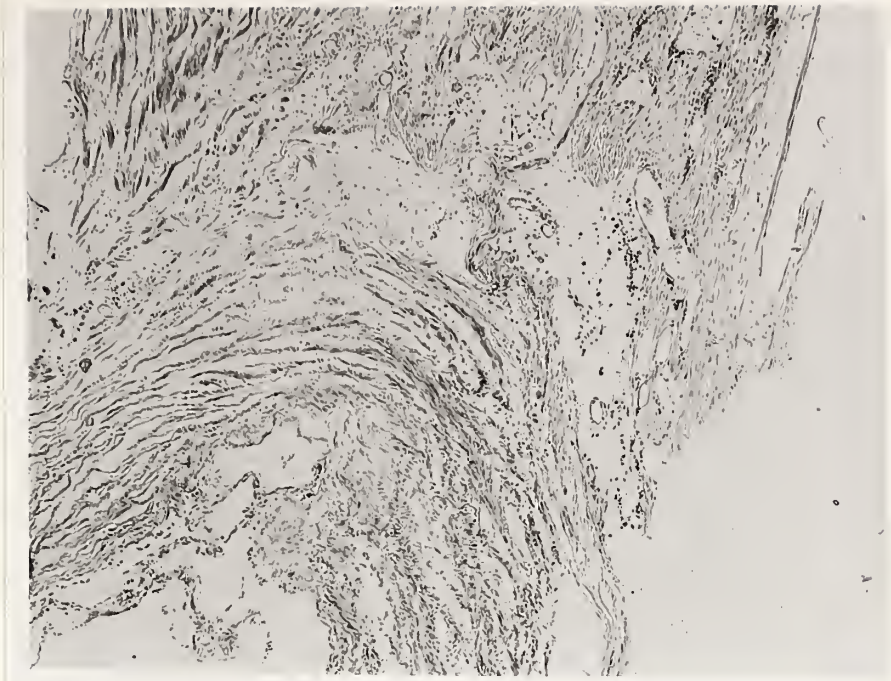

FIG. 13 - TRANSECTION SITE AT 12 WEEKS; 6 MICRONS; HEMATOXYLIN AND EOSIN X50.

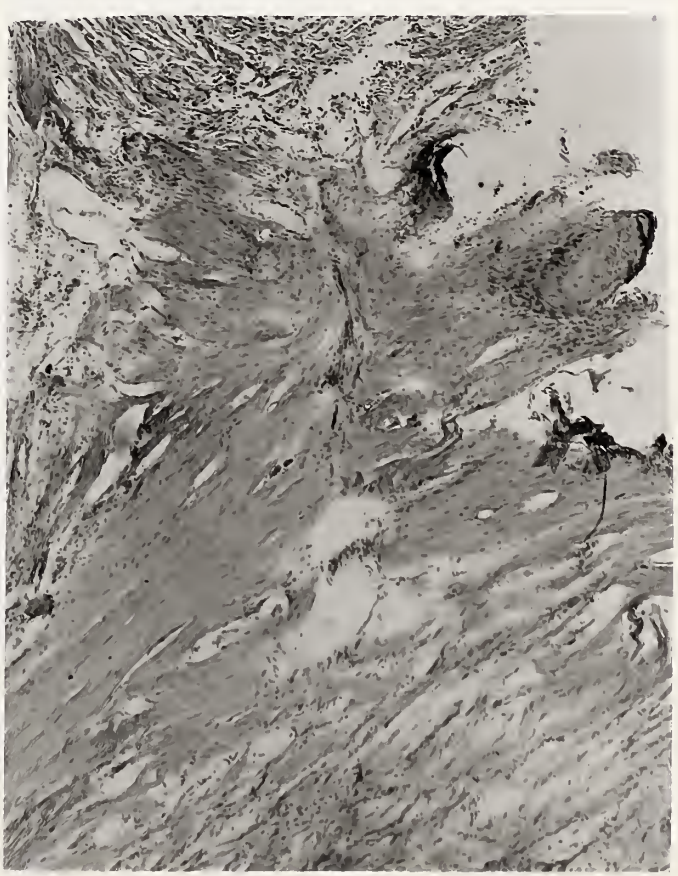

FIG. 14 - DENSE, TRANSVERSE CONNECTIVE TISSUE AT TRANSECTION SITE (20 WEEKS); 8 MICRONS; HEMATOXYLIN AND EOSIN X50.

portions of the neuroglial scar and the proximal connective tissue scar. As the neurofibrils approached the central area of the scar where the connective tissue was transversely-oriented, they were turned from their longitudinal course. Many grew back towards the spinal cord stump from which they had originated. No neurofibrils were seen to have entered or to have crossed the mid-zone of the transection site. This zone was occupied by relatively dense, transverse fibrous tissue. After the fourth week postoperatively, decreased numbers of regenerating neurofibrils were observed beyond the ends of the transected spinal cord. Organization of both the mesodermal and neuroglial scars was well-advanced. A few neurofibrils could occasionally be seen in the lateral aspects of the connective tissue scar. These neurofibrils may have originated from the dorsal roots of the spinal cord as there was no evidence of continuity with central nervous axons. By 9 weeks postoperatively, all signs of axonal regeneration were absent. The final appearance at the transection site was one of a dense, transverse, constricted connective tissue scar. The failure of axonal regeneration histologically agreed with the complete absence of functional and electrophysiological evidence of spinal cord regeneration.

B. EVALUATION OF TREATMENT WITH METHYL PREDNISOLONE

Weight change following methyl prednisolone administration was found to be a reliable index of the duration of action of the drug in the rat. Normal 150 Gm. rats gained approximately

5 Gm. per day until a weight of 180-190 Gm. was reached, and thereafter gained approximately 3 Gm. per day. After methyl prednisolone administration in Group II(a), an initial period of weight loss was followed by a period during which the rate of weight gain was less than normal. The effect on weight depended on the amount of drug administered. In the first two days following low-dosage steroid injection, a decrease in weight of up to 10 Gm. occurred. This weight was regained in 4 days following 0.2 mg. methyl prednisolone, 5 days (0.4 mg.), 8 days (0.6 mg.), or 9 days (1.0 mg.). When 8.0 mg. methyl prednisolone was administered, a weight loss of 30 Gm. occurred during the first 6 days. This loss was not regained by 13 days (FIG. 15).

Methyl prednisolone was found to delay wound healing in Group II(b). In untreated rats subjected to a mid-dorsal skin incision, Michel clips were removed 4 days postoperatively and separation of the skin edges did not occur. In rats receiving 0.4 mg. methyl prednisolone, skin separation after clip removal occurred at 2 and 4 days but not at 6 days postoperatively. After 1.0 mg., separation occurred at 8 but not at 10 days,while following 8.0 mg., firm union of the skin edges did not occur until 16 days postoperatively.

The effect of methyl prednisolone on the formation of scar tissue at the site of spinal cord transection was investigated in Group II(c). In an initial series of 80 rats, the

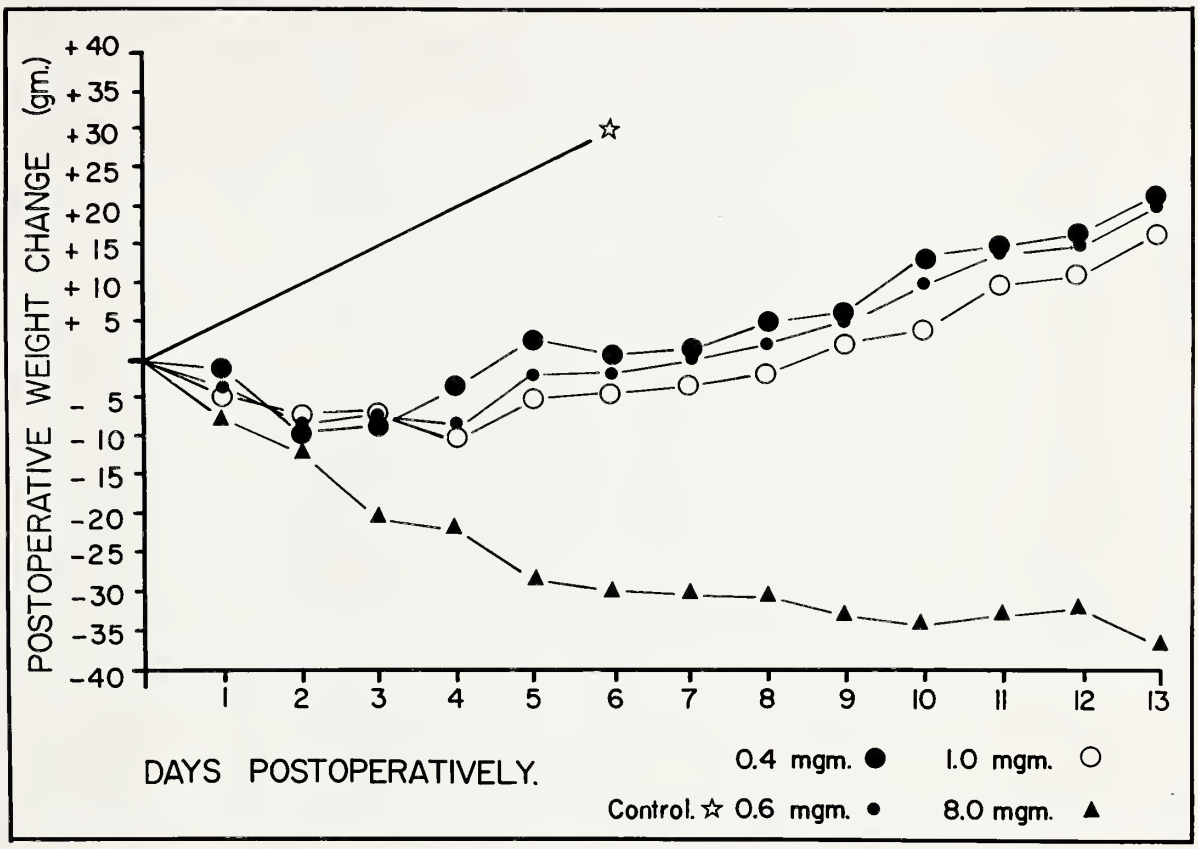

FIG. 15 - WEIGHT CHANGE IN UNOPERATED RATS FOLLOWING METHYL PREDNISOLONE ADMINISTRATION.

first dose of methyl prednisolone was injected at operation in an amount (8.0 mg.) which was associated with a marked delay in skin wound healing in Group II(b). Thereafter, the same amount was administered every 2 weeks, an interval which the results in Group II(a) indicated was well within the duration of therapeutic effect of the drug.

Complications developed in most of the rats within the first week. Failure to eat the commercial rat diet was associated, somewhat paradoxically, with a high incidence of cannibalism of the insentient feet and hindquarters. The cannibalism was not deterred in these rats by daily application of picric acid to the hindquarters. Massive hematuria was present in approximately one-third of the rats. Autopsy revealed large clots in the bladder lumen, hematomas in the bladder wall and mucosa, and normal kidneys. Early slight hematuria had been noted in control rats after cord transection, but the urine was clear after 1-2 days. The blood was attributed either to distention of the bladder between the times urine was expressed, or to trauma during expression. Cannibalism and hematuria, together with a high incidence of pneumonia, in this series, were associated with an 85% mortality (68 rats) in the first month postoperatively. The longest survival time was 10 weeks (2 rats). None of the rats showed evidence of return of motor or sensory function prior to sacrifice or death. Electrical stimulation of the brain stem and spinal cord was not

performed in this series. Cachexia was extreme in all of the rats (FIG. 16).

A second series of 30 rats was later prepared with spinal cord transection. Methyl prednisolone (2.0 mg.) was administered at operation and at 14 days postoperatively. Cannibalism was infrequent. Hematuria, when it occurred, was moderate and cleared spontaneously. The majority of the rats, however, developed pneumonia postoperatively; these were either sacrificed or died spontaneously. The rest were sacrificed at or before 3 weeks postoperatively for histological examination of the spinal cord. None of the rats showed evidence of return of motor or sensory function prior to sacrifice or death. Electrical stimulation studies were not conducted in this series.

The third series treated with methyl prednisolone consisted of 75 rats. The spinal cord was completely transected and 0.2 mg. methyl prednisolone was injected at operation, followed by 0.2 mg. at 14 days postoperatively and 1.0 mg. every 2 weeks thereafter. The mortality in the first month was approximately 60% (44 rats). The major cause of death was again pneumonia, although rats were sacrificed at intervals for histological examination of the spinal cord. No rats in this series developed coordinated walking or evidence of return of sensation. Functional performance differed in no way from that of rats with a segment of cord excised. Electrical stimulation of the brain stem and spinal cord above the transection

FIG. 16 - SEVERE WASTING IN A RAT AFTER ADMINISTRATION OF 8.0 mg. METHYL PREDNISOLONE EVERY 14 DAYS FOR 6 WEEKS; CONTROL RAT OF SAME AGE SHOWN ON LEFT; BOTH RATS PARAPLEGIC FOR 6 WEEKS.

site was performed in 18 of the rats surviving more than one month postoperatively, but no movements of the hind limbs could be elicited.

The results of histological examination of the spinal cord of the rats in Group II(c) showed that methyl prednisolone alters the appearance of the connective tissue scar at the site of spinal cord transection. The effect on connective tissue was found to be not on the cellularity of the scar, but on the formation of collagen fibers. Maximal effects on collagen formation were seen with doses of 8.0 mg., while a lesser effect was noted with 2.0 mg. Methyl prednisolone in a dosage of 0.2 mg. at operation and at 14 days postoperatively did not exert an appreciable effect on scarring. However, when 1.0 mg. was administered at 28 days and every 2 weeks thereafter in this series, further collagen deposition appeared to be less than in controls.

Collagen formation in control rats began in the second postoperative week (7-14 days) and was most marked between 4 and 6 weeks after cord transection. Later collagen deposition occurred at a slower rate. In the series treated with 2 mg. methyl prednisolone at operation and at 2 weeks postoperatively, the early formation of connective tissue fibers seen in the control rats was not present. In the series treated with 8.0 mg. at operation and every 2 weeks thereafter, there was minimal formation of connective tissue fibers at 4 weeks (FIG. 17),

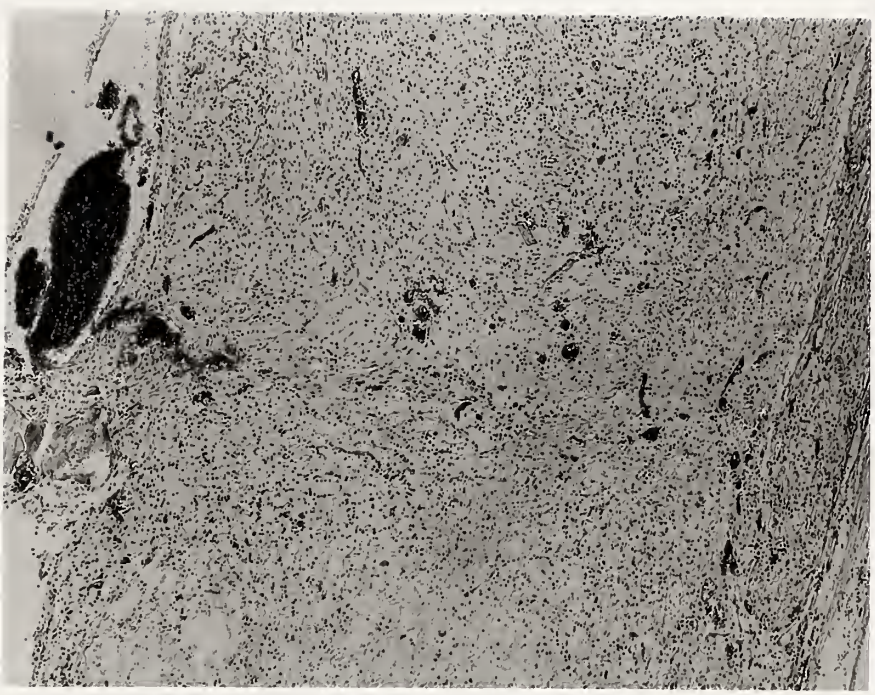

FIG. 17 - SPINAL CORD TRANSECTION SITE 4 WEEKS POSTOPERATIVELY IN A RAT TREATED WITH METHYL PREDNISOLONE.
8 MICRONS; HEMATOXYLIN AND EOSIN X50.

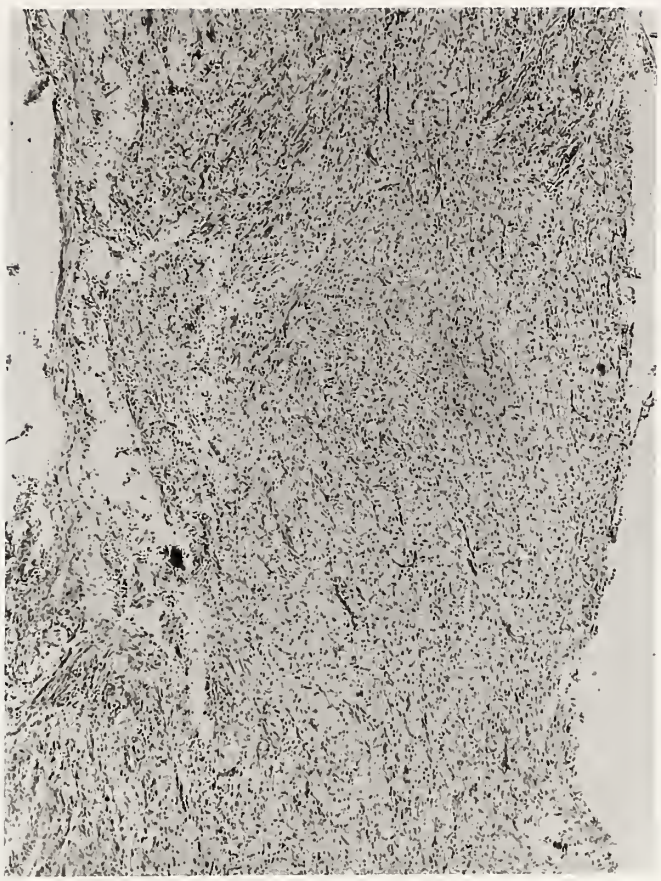

FIG. 18 - SPINAL CORD TRANSECTION SITE 5 WEEKS POSTOPERATIVELY IN A RAT TREATED WITH METHYL PREDNISOLONE.
8 MICRONS; HEMATOXYLIN AND EOSIN X50.

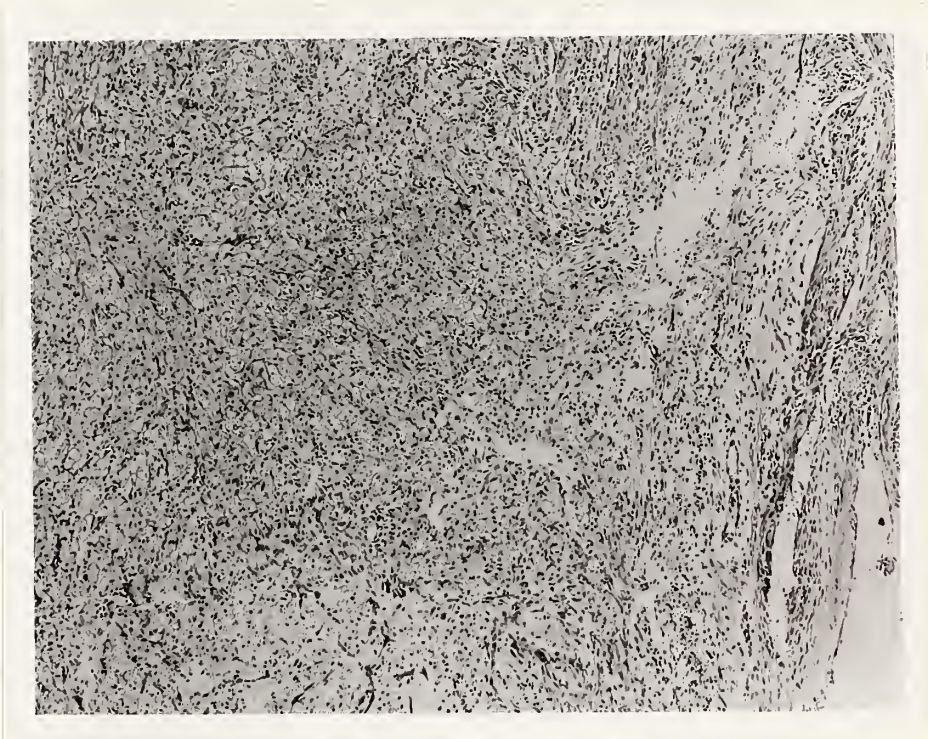

FIG. 19 - SPINAL CORD TRANSECTION SITE 6 WEEKS POSTOPERATIVELY IN A RAT TREATED WITH METHYL PREDNISOLONE.
8 MICRONS; HEMATOXYLIN AND EOSIN X50.

5 weeks (FIG. 18) and 6 weeks (FIG. 19). The mesodermal cells in the scar appeared immature and resembled those seen in the organizing blood clot 1 week postoperatively in the control rats. In the 8.0 mg. steroid series, these mesodermal cells were not associated with the formation of a significant amount of collagen. A few, thin, scattered fibers were evident, but there was no resemblance at 6 weeks to the heavily fibrous scar seen at that time postoperatively in the control rats. The transection site in rats treated with methyl prednisolone was well-vascularized.

The neuroglial scars over the stumps of the transected spinal cord tended to blend with the cellular mesodermal region. Otherwise, the appearance of the neuroglial portion of the scar was not altered by treatment with methyl prednisolone.

Elimination of the transverse fibrous scar during the period of maximal neurofibrillar growth was not associated with histological signs of complete central axon regeneration across the transection site. The time-course of neurofibrillar growth in rats receiving 8.0 mg. of methyl prednisolone was similar to that of untreated rats with spinal cord transection. Regenerating neurofibrils were observed in the glial scar and the adjacent part of the cellular mesodermal scar by 4 weeks postoperatively. None was seen in the central portion of the mesodermal scar. By 6 weeks, the only neuro-

fibrils demonstrable in the scar were sparsely distributed in the tissue immediately adjacent to the spinal cord stumps. No neurofibrils were found crossing the loose, cellular mesodermal tissue. When the dosage of methyl prednisolone was reduced to 0.2 mg., fibrous tissue formation increased. Neurofibrils tended to grow along the connective tissue strands, but no neurofibrils were seen in the central region of the scar.

The absence of histological evidence of spinal cord regeneration agreed with the negative functional and electrophysiological results in Group II(c).

C. EVALUATION OF TREATMENT WITH BACTERIAL PYROGEN

One of the three lots of Piromen tested chemically (see METHODOLOGY) for evidence of deterioration in storage was found to be inactive and was discarded.

The 10 unoperated rats in Group III(a) receiving 1 microgram of Piromen per day increased in weight at a rate comparable to untreated control rats. Unlike the steroid-treated rats in Group II, weight change could not be used as an index of either the biological activity of Piromen or the development of tolerance during prolonged administration.

The 75 rats in Group III(b) were subjected to spinal cord transection. Piromen, 1 microgram per day, was administered for 5 weeks. Mortality in the first month was approximately 50% (38 rats) as compared with 44% (33 rats) in 75 rats with spinal cord transection alone (Group I(c)). None of the

rats receiving Piromen developed coordinated walking or evidence of return of sensation distal to the transection site. The maximum period of observation was 17 weeks. No movements of the hind limbs resulted from electrical stimulation of the brain stem or spinal cord. Histologically, the spinal cord transection site in rats treated with bacterial pyrogen resembled that of untreated control rats. The cellularity and amount of fibrous tissue in the mesodermal scar was similar in the two groups. There was no discernible alteration of the neuroglial scars. The time-course of neurofibril appearance, growth and disappearance in the scar was comparable to that noted in rats not receiving Piromen. The absence of histological evidence of spinal cord axonal regeneration across the transection site agreed with negative functional and electrophysiological results.

D. EVALUATION OF ORGANOTHERAPY ALONE AND IN COMBINATION WITH CHEMOTHERAPY

The 75 rats in Group IV(a) received injections of central nervous tissue homogenate without concomitant chemotherapy following spinal cord transection. By 6 weeks postoperatively, 40 of the 75 rats had died or were sacrificed for histological studies of the transection site. Death in the homogenate-treated rats was most commonly associated with urinary tract infection. Autopsy revealed that in many of the rats the urinary bladder was distended with cloudy urine and many small calculi. Bladder calculi, with subsequent lower urinary tract obstruction and infection, were seldom

seen in rats not receiving homogenate injections.

None of the 40 rats in Group IV(a) which died or were sacrificed prior to 6 weeks postoperatively had shown evidence of return of motor or sensory function distal to the transection site during life. There was no movement of the hind limbs which could not be explained on a reflex basis. However, although functional evidence of spinal cord regeneration was absent, histological studies during the first 6 weeks revealed abundant evidence of axon regeneration at the transection site.

Histologically, the transection site in homogenate-treated rats resembled that of control rats up to 2 weeks post-operatively. Early connective tissue formation was present. Regenerating neruofibrils were observed in the neuroglial scar and the adjacent part of the connective tissue scar. The neurofibrils arose from both the proximal and distal spinal cord stumps.

By 3 weeks postoperatively (FIG. 20), all regions of the mesodermal scar at the transection site contained neuro-fibrils (FIG. 21) in the homogenate-treated rats. In control rats the central region of the scar contained no demonstrable neurofibrils 3 weeks after operation. The amount of meso-dermal and neuroglial scarring was similar in the treated and untreated rats.

By 6 weeks postoperatively (FIG. 22) in the homogenate-treated rats, neurofibrils were still abundant in all parts of

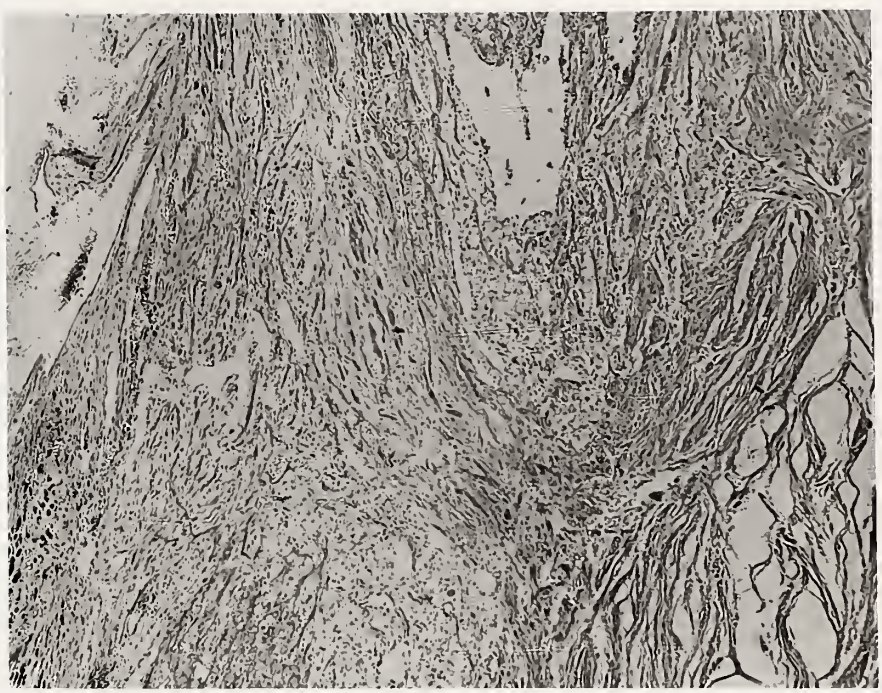

FIG. 20 - SPINAL CORD TRANSECTION SITE 3 WEEKS POSTOPERATIVELY IN RAT TREATED WITH NERVOUS TISSUE HOMOGENATE. BODIAN'S PROTARGOL METHOD X50.

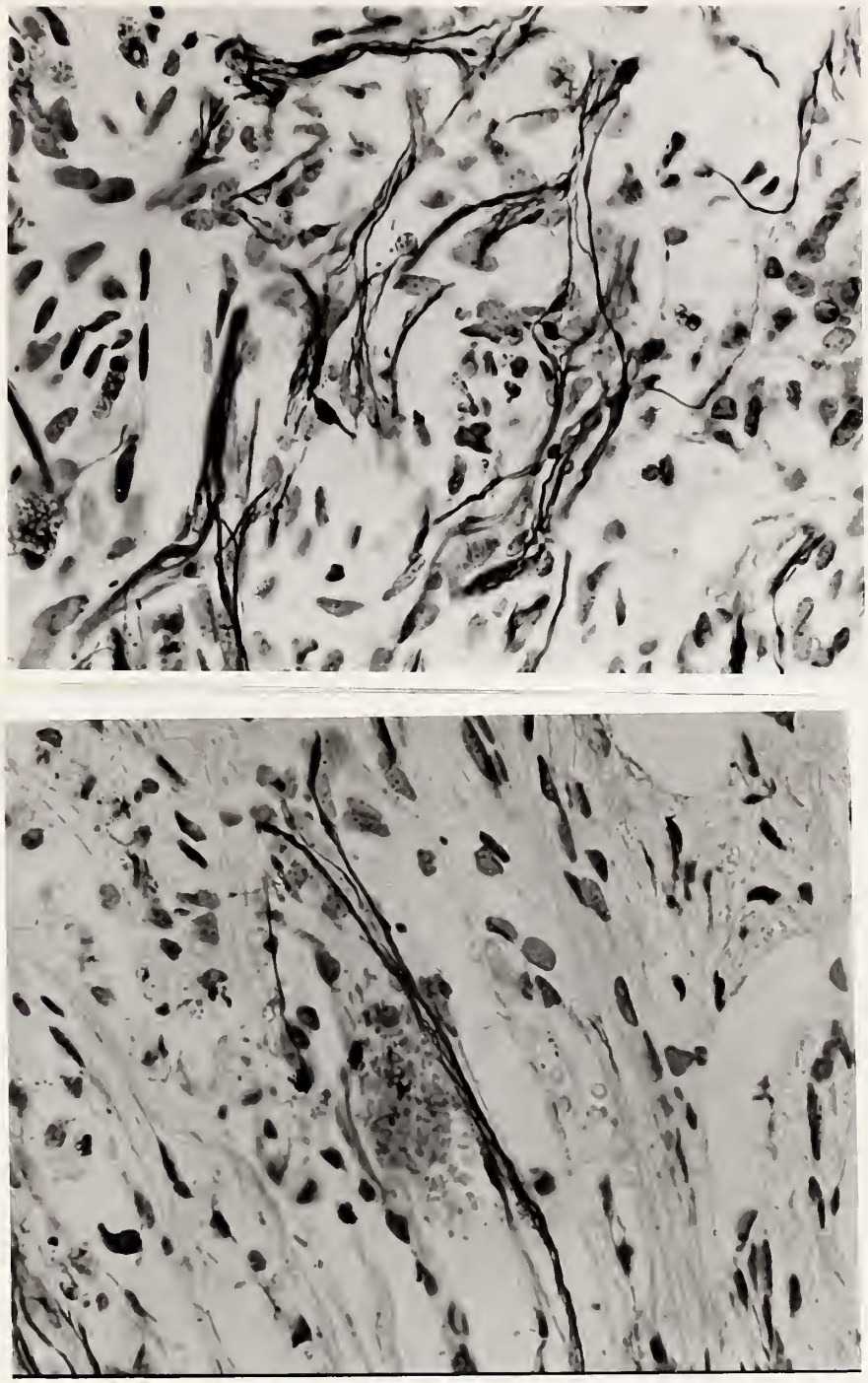

FIG. 21 - DETAILS FROM FIG. 20 SHOWING REGENERATING NEUROFIBRILS IN SCAR AT TRANSECTION SITE. BODIAN'S PROTARGOL METHOD X500.

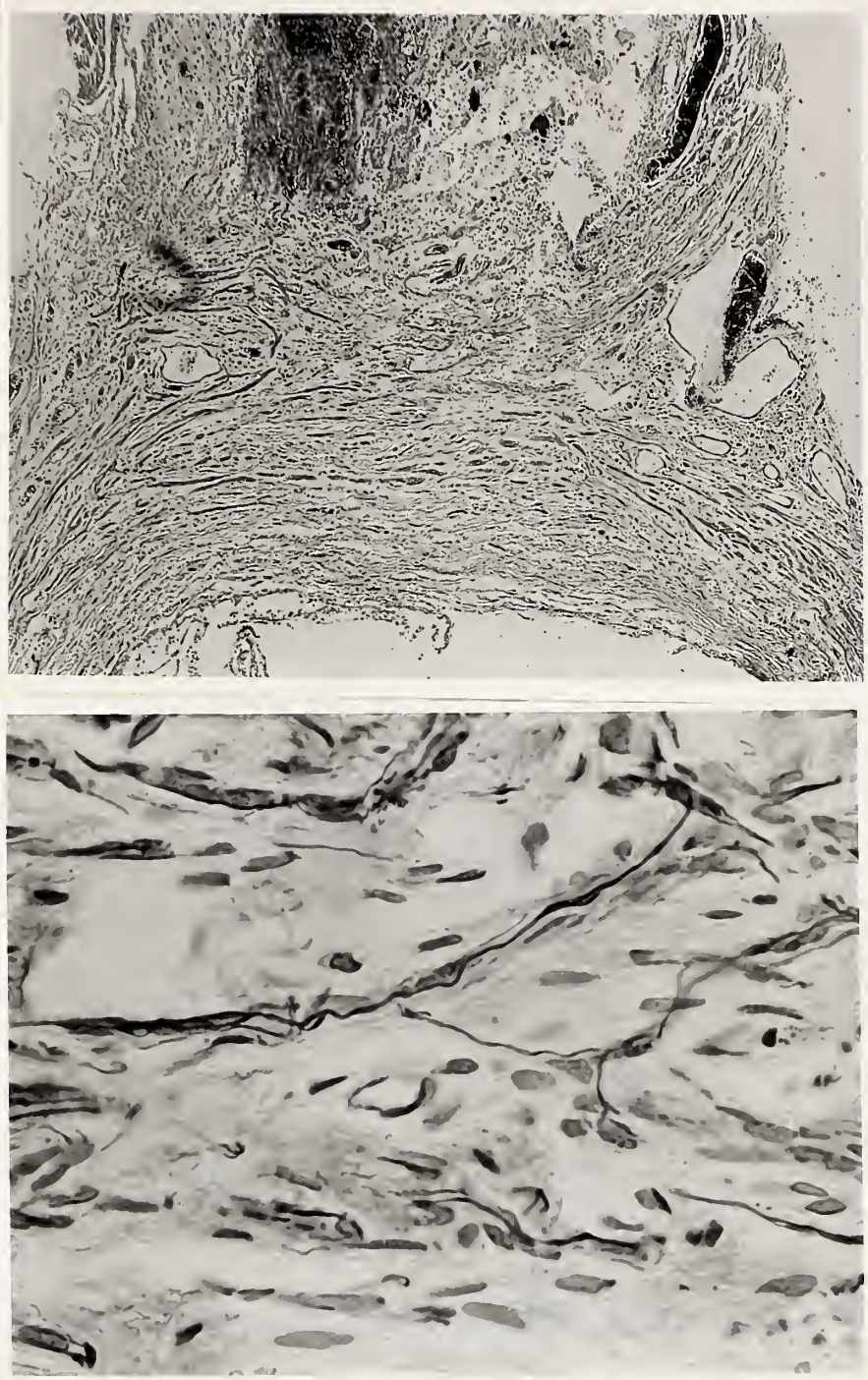

FIG. 22 - NEUROFIBRILS IN CENTRAL AREA OF SCAR 6 WEEKS POST-OPERATIVELY IN A RAT TREATED WITH NERVOUS TISSUE HOMOGENATE. BODIAN'S PROTARGOL METHOD X50 ABOVE, X500 BELOW.

the scar. In control rats (Group I(c)), few neurofibrils could be seen except in the neuroglial portion of the scar immediately near the cord stumps.

Of the 35 rats in Group IV(a) maintained for longer than 6 weeks postoperatively, 5 rats subsequently exhibited activity in the hind limbs which could not be ascribed to reflex phenomena.

Rat HF12 exhibited locomotor signs, differing from those of typical paraplegic rats, in the seventh week after spinal cord transection. The first sign to appear was a coordination of movement in the left leg and foot with that of the forelimbs when the rat climbed an inclined screen. The following day, the coordinated movements were observed when the rat moved across a level surface. In addition, the left foot was moved in a full arc beneath the bulk of the hindquarters. The toes were observed to splay as a backward thrust of the foot occurred. The thrust of the foot briefly lifted the hindquarters. By the end of the seventh week, spontaneous movements of the hind limbs were made while the rat was at rest and both hind limbs made coordinated movements during walking. The coordinated movements were present on a smooth, level surface. Over the next month, the weight of the hindquarters was increasingly supported by the legs and feet as the rat walked. Near-normal walking ability was regained. Injection of 1% procaine hydrochloride into the

spinal canal above the transection site abolished spontaneous movement in the hind limbs without abolishing reflex activity in the hindquarters. Rat HF12 was sacrificed 25 weeks after operation. There had been no discernible return of the sensations of pain, temperature or light touch distal to the transection site. Some degree of proprioception in the hindquarters was indicated by the hind limb movements while the rat was climbing. Placing and seeking of the feet and toes appeared normal. However, when the rat was made to grasp a horizontal suspended bar with the forepaws, and the bar was lowered so that the rat's feet and hindquarters touched a level surface, the rat would not release its grip on the bar (normal rats drop to a surface which the feet, legs or tail are touching).

Rat HF14 began to exhibit coordinated walking in the seventh week postoperatively. The movements were first noted in the rat's left leg, although coordinated walking movements later developed in the right leg. There was no demonstrable return of sensation distal to the transection site during the 23-week observation period. Rat HF14 developed pneumonia and died. The spinal cord was recovered after death, but attempts to stain for neurofibrils were unsuccessful.

Rats HF37, HF41 and HF18 first showed coordinated walking movements in the hind limbs in the eighth week after spinal cord transection. Only rat HF37 supported the full weight of the hindquarters on the feet during forward locomotion.

In rats HF41 and HF18 the abdomen intermittently scraped the surface over which the rat was walking. No signs of return of sensation could be elicited in any of the 3 rats. Rat HF37 was maintained for 22 weeks. Prior to sacrifice, electrical stimulation of the brain stem and spinal cord above the transection site produced a fine tremor of the hind limbs in time with the stimulation frequency (5 pulses per second, duration 1 millisecond, strength 1 volt). Rat HF18 ceased breathing during electrical stimulation of the brain stem and spinal cord, 24 weeks after operation. Agonal movements of the hind limbs supervened, and no unequivocal hind limb response to electrical stimulation could be detected. Rat HF41 was sacrificed 25 weeks after spinal cord transection. The results of histological study of the spinal cords of rats HF12, HF18, HF37 and HF41 are shown in FIG. 23-26.

The other 30 rats in Group IV(a) which were maintained for longer than 6 weeks after spinal cord transection did not differ functionally from rats with a segment of spinal cord excised. There was no evidence of return of sensation in the hindquarters. Maximum movement in the legs consisted of short, rhythmic step-like motions of the feet as the hindquarters were dragged up or along a rough surface (wire screen). There was no active participation of the toes in forward locomotion. The weight of the hindquarters was not supported to any extent whatsoever on the feet.

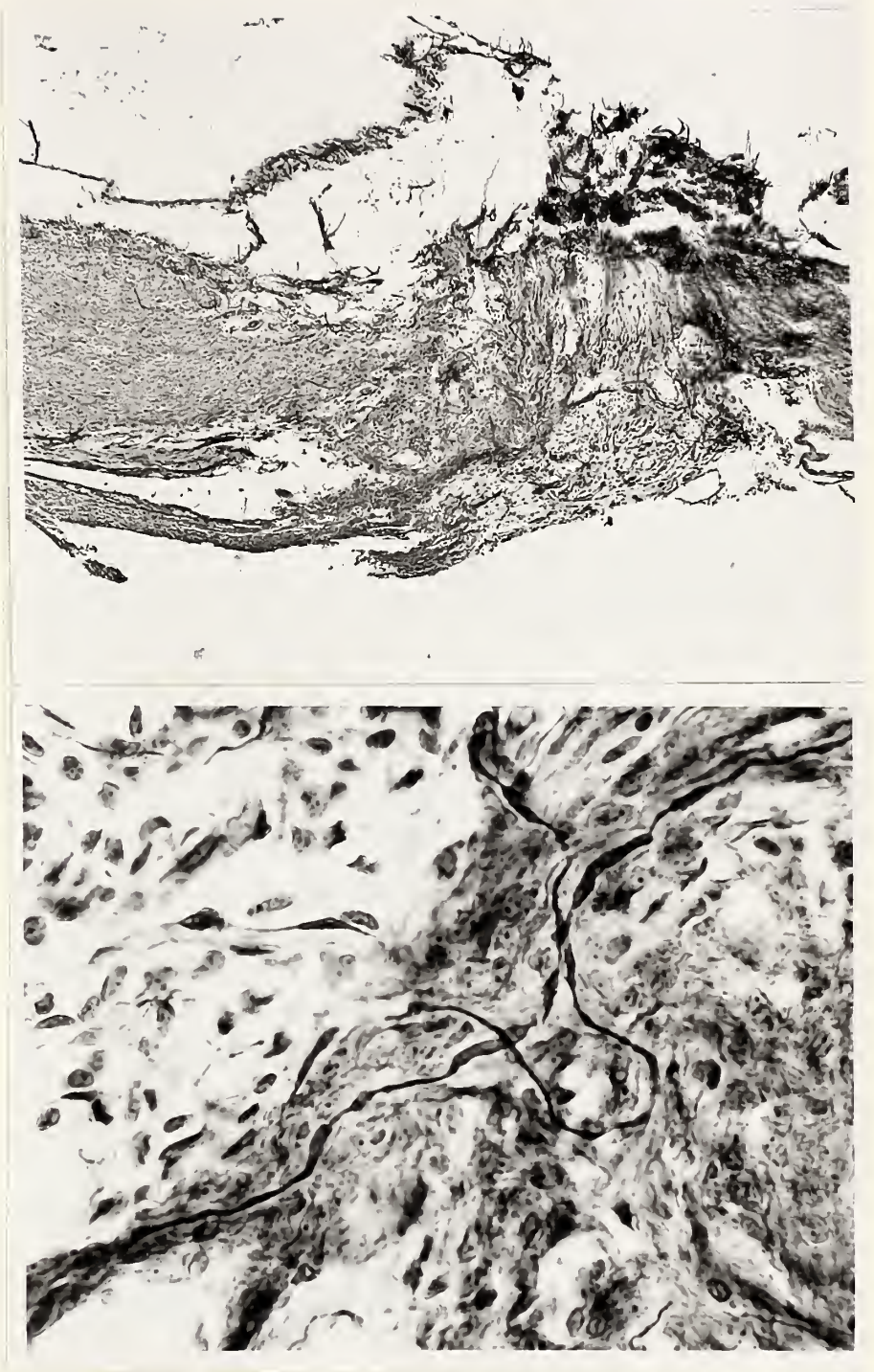

FIG. 23 - AXONS IN SCAR 25 WEEKS AFTER SPINAL CORD TRANSECTION IN A RAT (HF12) TREATED WITH NERVOUS TISSUE HOMOGENATE. MODIFIED BODIAN'S PROTARGOL METHOD; MAGNIFICATION X50 ABOVE, X500 BELOW; SECTION 8 MICRONS.

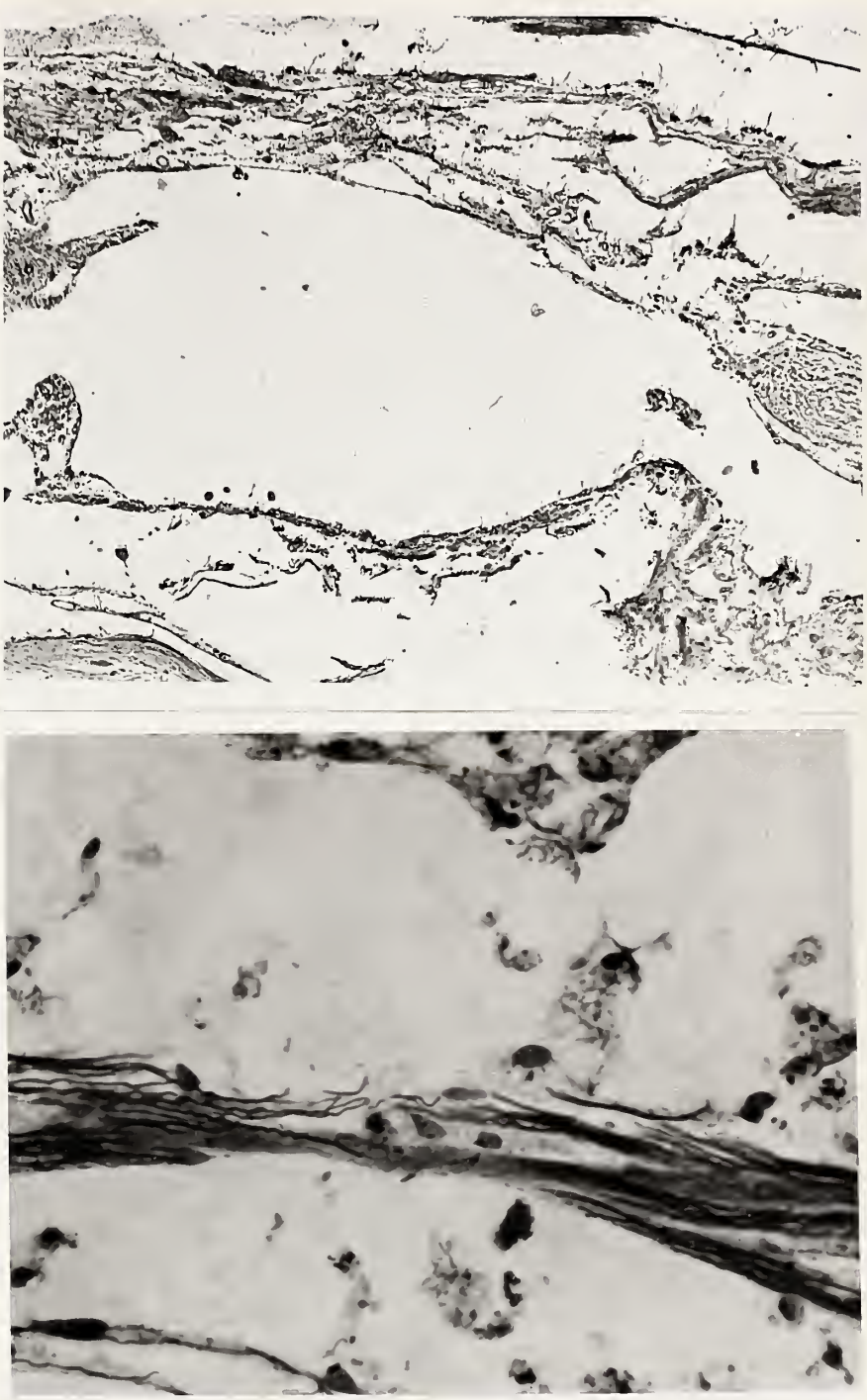

FIG. 24 - AXONS IN SCAR 24 WEEKS AFTER SPINAL CORD TRANSECTION IN A RAT (HF18) TREATED WITH NERVOUS TISSUE HOMOGENATE. MODIFIED BODIAN'S PROTARGOL METHOD; MAGNIFICATION X50 ABOVE, X500 BELOW; SECTION 8 MICRONS.

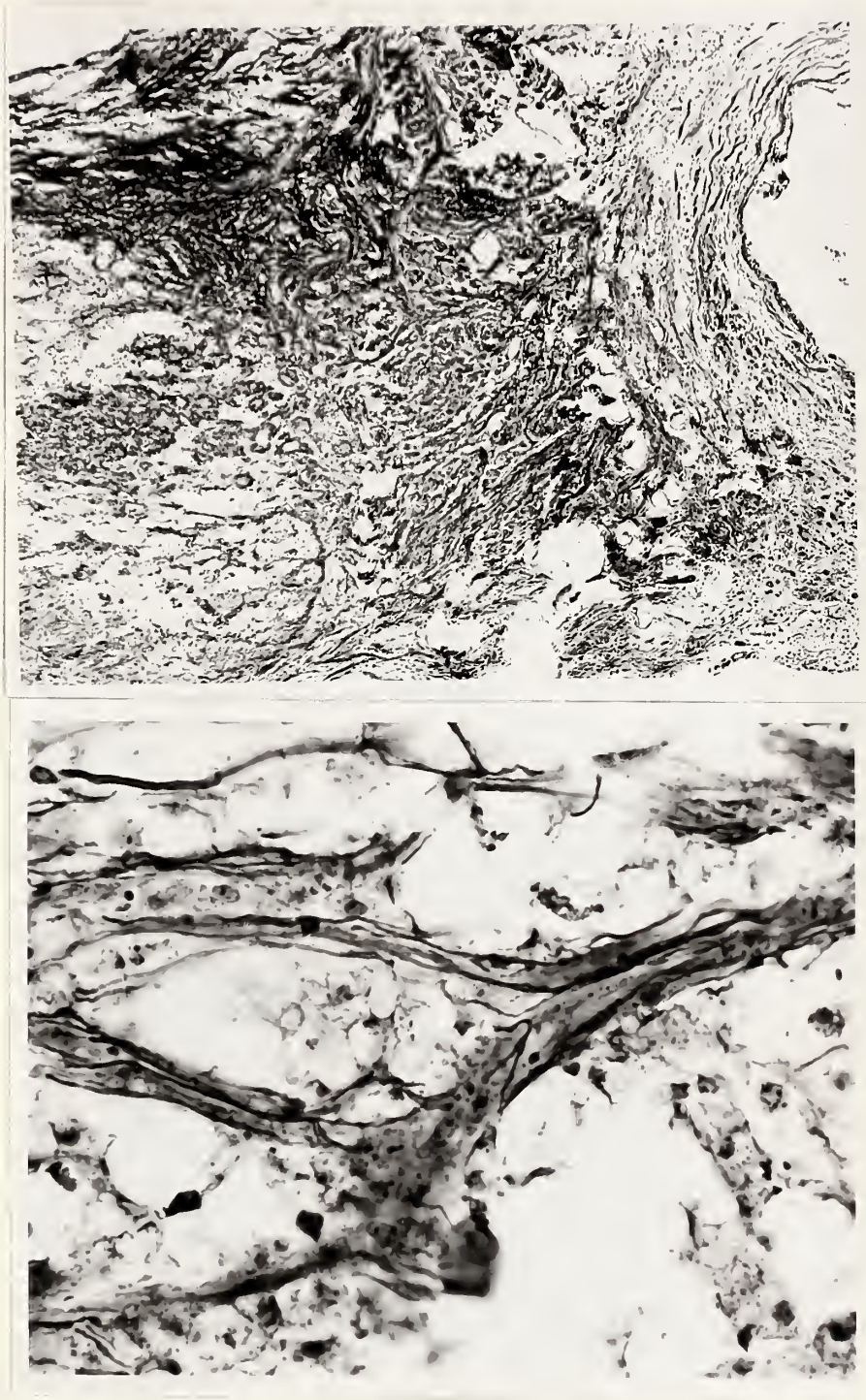

FIG. 25 - AXONS IN SCAR 22 WEEKS AFTER SPINAL CORD TRANSECTION IN A RAT (HF37) TREATED WITH NERVOUS TISSUE HOMOGENATE. MODIFIED BODIAN'S PROTARGOL METHOD; MAGNIFICATION X50 ABOVE, X500 BELOW; SECTION 8 MICRONS.

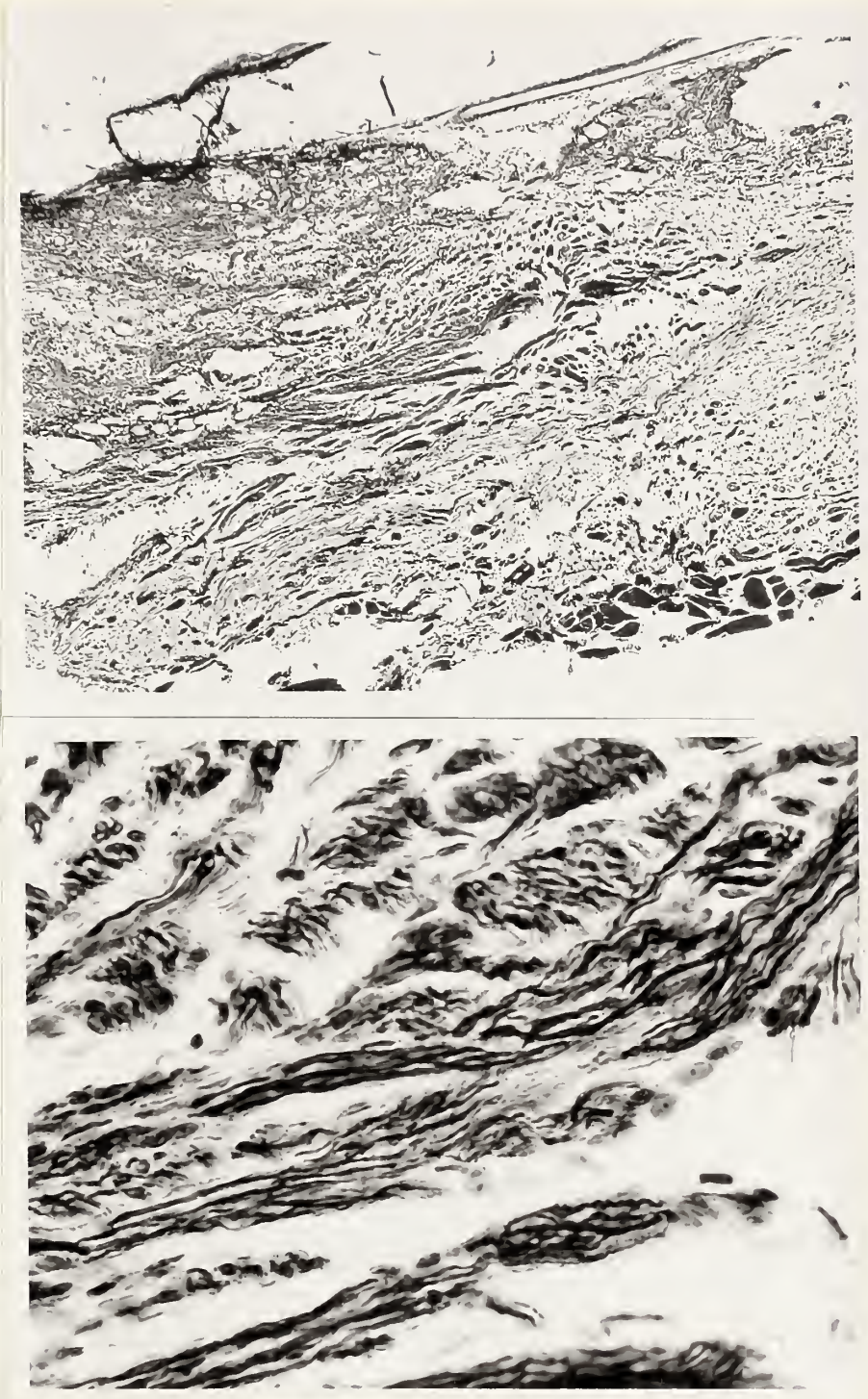

FIG. 26 - AXONS IN SCAR 25 WEEKS AFTER SPINAL CORD TRANSECTION IN A RAT (HF41) TREATED WITH NERVOUS TISSUE HOMOGENATE. MODIFIED BODIAN'S PROTARGOL METHOD; MAGNIFICATION X50 ABOVE, X500 BELOW; SECTION 8 MICRONS.

In Group IV(b) 30 rats received nervous tissue homogenate plus bacterial pyrogen (Piromen). Deaths in the first 6 weeks after spinal cord transection were associated with pneumonia (4 rats), cannibalism of the feet (6 rats), urinary tract infection (3 rats) and sacrifice for histological study (8 rats).

Of the 9 rats surviving to 6 weeks postoperatively, 1 rat (PH25) exhibited spontaneous movements of the hind limbs. Coordinated movements of the front and hind limbs, with active participation of the toes, were first noted 43 days after spinal cord transection. The weight of the hindquarters was lifted slightly and alternately by the legs as the rat walked, but the rat could not stand unaided. No signs of recovery of sensation could be elicited. On the 44th postoperative day, the rat developed urinary retention and was sacrificed to preserve the spinal cord for histological examination. Prior to sacrifice, electrical stimulation of the cervical spinal cord produced movements of the hind limbs in time with the stimulation frequency (2 pulses per second, strength 1 volt, duration 1 millisecond). The remaining 8 rats in Group IV(b) were all maintained for 16 weeks after cord transection. None had developed coordinated walking ability or shown evidence of return of sensation by the end of the observation period.

Microscopically, the appearance of the spinal cord transection site in the rats in Group IV(b) treated with both

bacterial pyrogen and central nervous homogenate differed from that of the control (Group I(c)) and pyrogen-treated (Group III(b)) rats. Many small blood vessels were present throughout the scar by 2 weeks after spinal cord transection in Group IV(b). The mesodermal scar showed cellularity and fibrous tissue formation between the cut cord ends which was similar to the control group. There was no reduction of the neuroglial scarring. Regenerating neurofibrils with terminal growth bulbs were seen in all regions of the mesodermal scar 3 weeks after spinal cord transection. Many of these nerve processes were observed to have arisen from one or the other spinal cord stump. None, however, could be traced through the length of the scar. The net effect of the combined pyrogen-homogenate therapy by 6 weeks appeared to be the formation of a mesodermal lattice-work densely infiltrated with neurofibrils. Neurofibrils were abundant in the transection site scar at 44 days in the walking rat PH25 (FIG. 27).

In Group IV(c), central nervous tissue homogenate administration was combined with methyl prednisolone therapy. The purpose of the dual treatment in this group of 45 rats was to study the effect of homogenate on axon regeneration when an attempt was made to reduce the formation of connective tissue fibers in the scar. None of the rats survived longer than 3 weeks postoperatively. Pneumonia, urinary tract infection, self-cannibalism and rupture of the urinary bladder

accounted for some of the deaths. Many rats in this series, however, died without a probable cause of death being determined. Electrical stimulation studies were not conducted in Group IV(c). Locomotor or sensory function did not return in any of the rats prior to death or sacrifice. The short-term histological study (3 weeks) produced no conclusive results.

The final group of rats to be studied was Group IV(d). In 30 rats, homogenate and pyrogen were administered during the first 28 days following spinal cord transection. Deaths in the first 28 days were associated with pneumonia (10 rats), cannibalism of the feet (7 rats), urinary tract infection (3 rats) and rupture of the urinary bladder (1 rat). The 9 remaining rats were treated with 1.0 mg. methyl prednisolone on the 28th day, and every 2 weeks thereafter. Seven of these rats were maintained for 16 weeks after spinal cord transection. None showed a return of motor or sensory function distal to the mid-dorsal spinal cord transection site. Electrical stimulation studies were negative. Histologically, collagen fiber formation at the transection site appeared less dense than in control rats at the same date postoperatively, but neurofibrils could not be traced across the scar.

E. MORTALITY DATA

The number of deaths in each series of rats subjected to either spinal cord transection or segmental cord resection is summarized in TABLE I.

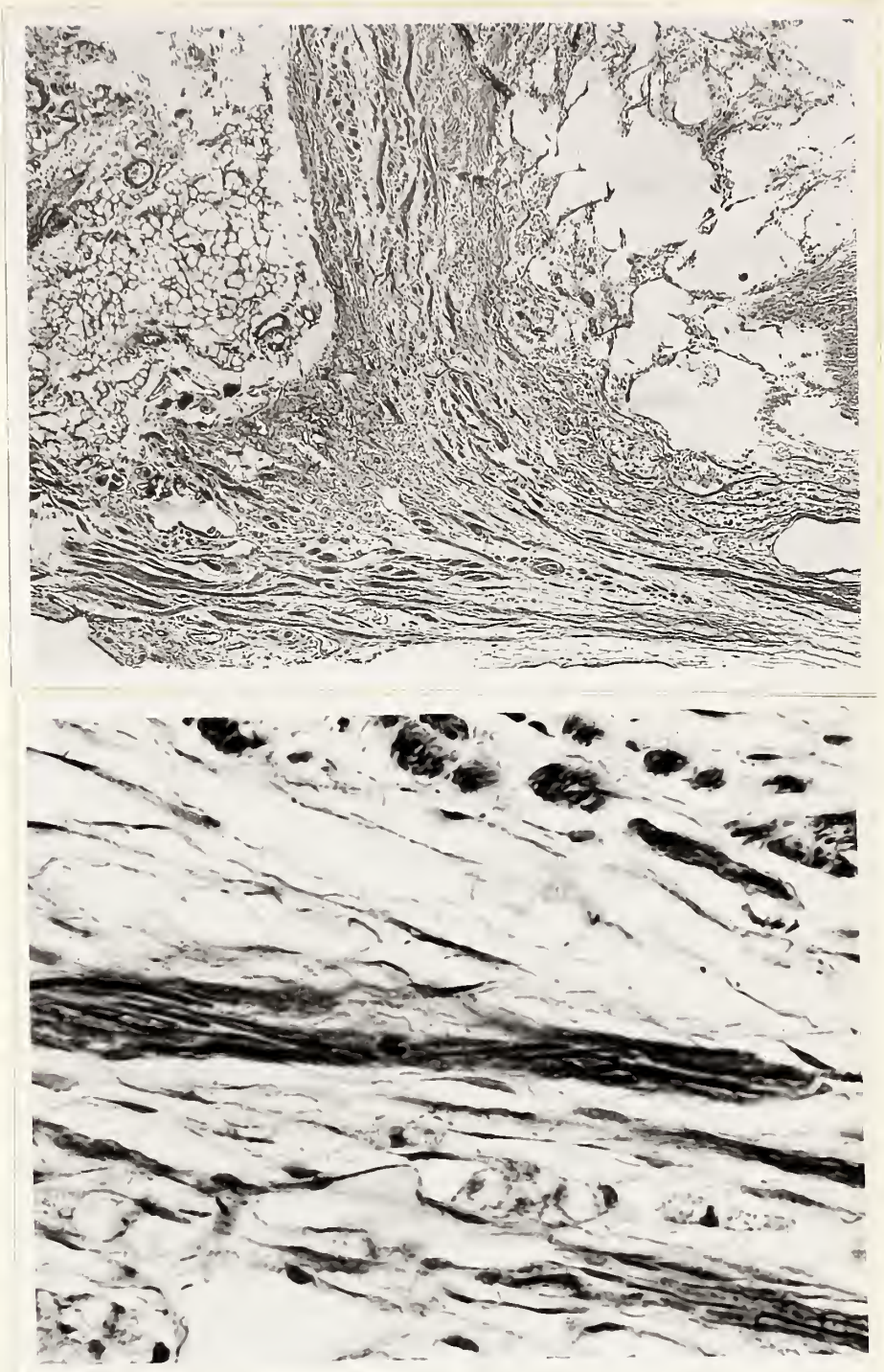

FIG. 27 - TRANSECTION SITE 44 DAYS POSTOPERATIVELY IN A RAT (PH25) TREATED WITH PIROMEN AND NERVOUS TISSUE HOMOGENATE. MODIFIED BODIAN'S PROTARGOL X50 ABOVE, X500 BELOW.

F. FUNCTIONAL DATA

A general summary of the results of the functional studies is presented in TABLE II.

TREATMENT	GROUP	SPINAL CORD OPERATION	NO. RATS OPERATED	NUMBER RATS DYING OR SACRIFICED POSTOPERATIVELY				+ 16 WEEKS
				0-4 WEEKS	4-8 WEEKS	8-12 WEEKS	12-16 WEEKS	
NIL	I(c)	TRANSECTION	75	33	8	6	3	25
NIL	I(d)	RESECTION	50	15	8	5	1	21
METHYL PREDNISOLONE	II(c)	TRANSECTION						
8 mg.			80	68	9	3	0	0
2 mg.			30	30	0	0	0	0
0.2 mg.			75	44	12	5	1	13
PIROMEN	III(b)	TRANSECTION	75	38	10	6	5	16
HOMOGENATE	IV(a)	TRANSECTION	75	31	15	7	0	22
HOMOGENATE + PIROMEN	IV(b)	TRANSECTION	30	19	3	0	0	8
HOMOGENATE + M.PRED.	IV(c)	TRANSECTION						
8 mg.			30	30	0	0	0	0
2 mg.			15	15	0	0	0	0
HOMOG.+ PIR.+ M.PRED.	IV(d)	TRANSECTION	30	21	2	0	0	7
TOTAL			565	344	67	32	10	112

TABLE I - MORTALITY DATA.

GROUP	NUMBER RATS OPERATED	NUMBER RATS MAINTAINED + 6 WEEKS	COORDINATED WALKING	SENSORY RETURN	NOTES
I(c)	75	38	0	0	-
I(d)	50	31	0	0	-
II(c)	185	31	0	0	-
III(b)	75	32	0	0	-
IV(a)	75	35	5	0†	HF12, 14, 18, 37, 41
IV(b)	30	9	1	0†	PH25
IV(c)	45	0	0	0	-
IV(d)	30	8	0	0	-

† Possible proprioception.

TABLE II - FUNCTIONAL DATA.

CHAPTER IV

D I S C U S S I O N

A. THE CRITERIA FOR SPINAL AXON REGENERATION

In an investigation of regeneration in the spinal cord, the first and most important objective must be the attainment of complete transection of all the spinal axons at the cord level chosen for study. If intact axons remain at the operative site, misleading experimental results are obtained and therapeutic measures cannot be properly evaluated.

In the extensive literature dealing with regenerative phenomena in the mammalian central nervous system, the reports of two groups of investigators merit detailed consideration. The published data of Sugar and Gerard (1940) and of Freeman and his co-workers (1949, 1952, 1955, 1962) are noteworthy in that both groups used essentially the same method of spinal cord transection and both obtained similar results. After repeating Sugar and Gerard's methods, Barnard and Carpenter (1950) concluded that attempting to cut the spinal cord by scalpel alone, maintaining the blade tip in continuous contact with the bony vertebral canal, was inadequate to ensure complete cord transection in all cases. Nevertheless, Freeman (1955) stated "the technique of cutting the cord and its investing membranes circumferentially by maintaining contact with the bone with a cataract knife or dagger blade" was employed as the method of choice for cord transection in his series of rats. The result was that "about 100 rats regained walking ability closely approximating that of the normal and about

twice that number had some degree of functional return" (Freeman. 1955). Freeman suggested that meticulous postoperative care was responsible for the observation of functional return in the rats.

Freeman's (1955) method of spinal cord transection was repeated by Speakman and Cseuz (1962) in 258 rats. Over half (52.3%) of the rats surviving longer than one month post-operatively showed coordinated walking, with many of the rats exhibiting evidence of return of sensation distal to the cord transection site. Histological examination of the spinal cords of the "recovered" rats revealed that the spinal cord had been incompletely transected. Residual intact axons evidently accounted for the functional return. Freeman's (1952, 1955) method of cord transection was seriously questioned as a means of ensuring severence of all the spinal axons.

In the present study, a curved, blunt probe was passed extradurally around the spinal cord before the transection with scalpel or iridectomy scissors. Complete transection of the spinal cord was denoted by unimpeded passage of the curved probe through the transection site. Freeman's (1955) objection that passing a blunt hook around the spinal cord before transection produced severe damage to the cord was shown to be invalid by the results in Group I(a) in the present investigation. Rats walked normally and exhibited unaltered sensory function within 5-15 minutes after the probe was passed completely

around the spinal cord and then removed. When use of the probe was omitted before transection, in Group I(b), several instances of incomplete transection of the spinal cord resulted. Therefore, passage of the extradural probe was made standard operative procedure in all rats subsequently subjected to cord transection. No further instances of incomplete transection of the spinal cord were encountered. Histological study revealed a complete scar across all of the cords examined.

After proof of complete severence of the spinal cord, the second criterion of spinal axon regeneration is the demonstration of clinical signs of restitution of motor and/or sensory function. Five rats in Group IV(a) and one rat in Group IV(b) exhibited locomotor ability sufficiently different from that of control rats (Group I(c), Group I(d)) and similar to that of normal rats to justify the assumption that a degree of motor function had returned.

The absence of functional evidence of sensory recovery in the rats in the present investigation contrasts with the sensory recovery claimed by Sugar and Gerard (1940) and Freeman (1952). The rats in the present study did not respond to noxious stimuli in the hindquarters. However, the development of coordinated walking in 6 of the rats seemed accompanied by proprioceptive ability in the hind limbs and feet. The present methods of clinical testing do not rule out the craniad passage of non-noxious sensory stimuli.

The third criterion for the demonstration of central

axonal regeneration is evidence of nervous conduction across the transection site following electrical stimulation. One rat in Group IV(a) - HF37 - and one rat in Group IV(b) - PH25 - exhibited movements of the hind limbs when the brain stem or cervical spinal cord were stimulated. The hind limb movements were in time with the stimulation frequency. The results in rat HF18 during electrical stimulation were equivocal as agonal movements in the hind limbs supervened. Rats HF12, HF14 and HF41 were not subjected to electrical stimulation.

The fourth criterion of regeneration is evidence that the observed motor activity is not solely on a spinal reflex basis. The paraplegic rat exhibits considerable reflex activity in the hindquarters. The rhythmic stepping movements in the feet and legs during the rat's forward locomotion often appear, when first seen,to be voluntary, but reflex activity can be demonstrated by several functional tests. The stepping movements increase when the rat climbs a coarse, inclined screen and disappear when the rat moves across a smooth, level surface. The toes are never used actively during forward locomotion. The weight of the hindquarters is never supported on the feet. The movements persist when procaine is injected intraspinally above the transection site.

Rats HF12, HF14, HF18, HF37, HF41 and PH25 all exhibited coordinated movements of the front and hind limbs during forward locomotion. The movements persisted when the rats walked across

a smooth level surface. All used the toes actively during walking and supported, to a varying extent, the weight of the hindquarters on the legs and feet. Spontaneous movements of the hind limbs occurred when the rats' forelimbs were immobilized. The objection raised by Hooker and Nicholas (1930) that tension on the rat's flanks or on the cord scar may induce reflex movements in the hind limbs seems invalid when the results of the present study are reviewed. Only 6 rats of the 565 rats subjected to spinal cord transection or segmental resection exhibited coordinated walking. None of the other rats showed similar signs, although these rats had well-developed reflexes and would, ostensibly, all have "flank tension". If tension were the cause of motor activity, then the intraspinal injection of procaine in rat HF12 should not have affected either tension on the cord scar or in the flanks. The procaine resulted, however, in the abolition of spontaneous movement in the legs and feet, although reflex movements remained intact.

The fifth and final criterion for the demonstration of axon regeneration in the mammalian spinal cord is the re-establishment of histological continuity of axons across the transection site. The neurofibrils and axons throughout the scar tissue at the transection site in rats treated with nervous tissue homogenate were seen to have originated from several sources. In the early scar, neurofibrils were observed

most commonly to be in continuity with the long fiber tracts in both proximal and distal cord stumps. Where a "retraction cyst" or cavitation had not developed at the transection site, neurofibrils with enlarged, bulbous ends were present in the scar adjacent to the grey matter. In the later scar, a dorsal root origin for some of the observed fibers cannot be completely eliminated as a possibility. However, no neurofibrils, or any origin, were seen in the scar of control rats after the sixth week postoperatively. In the homogenate-treated walking rats, where the axons could be traced for a distance in the scar, they appeared to have arisen from one or the other spinal cord stump. No fibers were seen to have arisen from severed dorsal roots.

B. THE INFLUENCE OF SCARRING ON AXON REGENERATION

The mesodermal scar has been postulated as the major barrier to spinal axon regeneration. However, Davidoff and Ransohoff (1948), Gokay and Freeman (1952), Clemente (1955), Freeman *et al* (1960) and Turbes, Freeman and Gastineau (1960) have all reported reduction in mesodermal scarring under various therapeutic regimens, but there has been little evidence of either permanent axonal regeneration or unequivocal functional return.

The results of the present study have shown that when collagen fiber formation in the scar is reduced and almost eliminated after methyl prednisolone administration, spinal

axon regeneration is not correspondingly enhanced. The transection site in rats treated with methyl prednisolone was better-vascularized than the scar in control rats, but the regenerating neurofibrils did not persist in the less-fibrous scar of the steroid-treated rats for any longer time (6 weeks) than they remained in the heavily collagenous scar of untreated rats. Axon regeneration began, and then ceased. Mesodermal scarring could not account for the observed sequence of events.

The neuroglial scar was thought to be a major barrier to regeneration by Clemente (1955). Claims that bacterial pyrogen reduced the glial scar and enabled axon regeneration were made by Windle and Chambers (1950), Clemente et al (1951), Scott and Clemente (1951), Gokay and Freeman (1952) and Littrell (1955). Negative results after bacterial pyrogen administration were reported by Bailey, Rooke and Rodin (1952), Lance (1954), Arteta (1956) and Windle et al (1956). Differences in response to Piromen among the different species of mammal tested may account in part for the negative results in rodents and primates. The majority of reports claiming reduction of the neuroglial scar with Piromen have come from investigators using cats and dogs.

Results in the present study have shown that the neuroglial cells over the proximal and distal spinal cord stumps did not present an insurmountable obstacle to the regenerating neurofibrils. No neurofibrils were seen to have been turned

back at the glial "barrier" towards the spinal cord stump from which they had originated. Most neurofibrils did, however, pursue a tortuous course among the neuroglial cells before entering the adjacent connective tissue portion of the scar. The observed lack of effect of the bacterial polysaccharide Piromen on the neuroglial tissue in Group III(b) was, therefore, of little consequence. Neurofibrils, with or without Piromen, did not persist in the scar.

C. THE INFLUENCE OF CNS HOMOGENATE ON SPINAL AXON REGENERATION

The present view on the failure of mammalian spinal cord axon regeneration was stated by Hess (1956) who concluded: "The nervous tissue of the mammalian central nervous system appears to lack inherently the ability to regenerate under ordinary circumstances". However, the somewhat less pessimistic statement of Ramon y Cajal (1928) should be recalled: "Irregenerability of the cord is not an immanent and fatal property of the neuronal architecture, but the result of the absence in the neuroglial scar of a nutritive, trophic and orienting environment similar to that produced in the peripheral stump of the nerves by the proliferated cells of Schwann".

Ramon y Cajal's suggestion for the future study of mammalian spinal cord regeneration seems to have been followed too literally by more recent investigators. The work of Harrison (1910) and of Penfield (1927) has been largely disregarded. Harrison (1910) showed that neurons will send out axonal processes in tissue culture in the absence of Schwann cells; he

considered that one of the necessary conditions of outgrowth is a medium which affords a solid support to the fibers. Penfield (1927) demonstrated that connective tissue scarring and glial proliferation were directly related to the amount of degenerating neural tissue (axoplasm, myelin) in a wound in the central nervous system. Despite the above considerations, recent investigators (Brown and McCouch. 1947; Barnard and Carpenter. 1949, 1950; Freeman. 1952) have introduced nerve grafts and central nervous tissue into the gap between the cord stumps in an attempt to enhance spinal axon regeneration. The result was, uniformly, a massive increase in scarring with no functional return.

The failure of spinal axon regeneration after the implantation procedures may not have been due solely to dense scarring, but also to the possibility that the "stimulating or nutritive substances" were applied at the wrong end of the nerve. Harrison (1910) showed that axon growth in culture is by an "ameboid-like" extension of the protoplasm of the cell soma. Weiss (1944) and Weiss et al (1945) showed that axoplasm dams proximally to a constriction in a nerve axon, and that marker substances deposited in nerves move distally along the axon. Further, Lewis (1945) considered that axoplasm is synthesized in the cell body and is forced along the axon to the developing end. In addition to confirming Harrison's tissue culture observations, Geiger (1958) noted a continuous streaming of

cytoplasm throughout the perikaryon, dendrites and axons of mammalian cortical nervous cells in culture. Also, transfer of nucleolar material to the cytoplasm was recorded.

The above observations strongly suggest that the material required for neurofibrillar outgrowth from transected axons is passed along the intact axon from the cell body and is produced, in part at least, in the neuron nucleus. Therefore, placing "nutritive substances" adjacent to the growing neurofibrils would have little, if any, beneficial effect if the materials for axoplasm synthesis, and hence neurofibrillar growth, were required in the distant cell soma. Also, the relative avascularity of the spinal cord transection site decreases the possibility that a significant absorption of these "nutrients" into the systemic circulation would occur.

The problem in the present investigation was to determine whether spinal cord axon regeneration could be enhanced or prolonged to a degree that functional return would occur distal to the site of cord transection. The results of spinal cord transection in the control rats (Group I(c)) indicated that spontaneous regeneration of the spinal cord does not occur in this mammal. Attempts to enhance neurofibril growth by reducing scarring at the transection site were unsuccessful in producing permanent regeneration of the spinal axons. Therefore, a method was sought for stimulating axoplasmic production and neurofibrillar growth indirectly through the neuron soma.

Many substances have been found to stimulate the regeneration of peripheral nerves *in vivo*, and to increase neurofibril production from sympathetic ganglia *in vitro*. Koechlin (1955) reported enhanced corneal re-innervation in rabbits after the daily intraperitoneal injection of extracts obtained from rabbit and calf brain. Levi-Montalcini (1955) reported that a diffusible material, from mouse sarcomas, was found to stimulate neurofibril outgrowth from sympathetic ganglia *in vitro*. May and Thillard (1954), using ganglion cultures, found axon-stimulating properties in an extract of degenerating peripheral nerve; however, the substance responsible for the growth effects was present in greater amounts in plasma alone than in peripheral nerve. An important contribution to the study of regeneration was made by Tumanishvili (1960) who found stimulation of regeneration of muscle and liver following the injection of tissue extracts. The extracts were not class-specific, but appeared to affect only homologous tissue in frogs, rabbits and guinea pigs.

In the present investigation, the material chosen for possible nerve growth-stimulating effects was a homogenate of central nervous tissue (brain and spinal cord) from newborn rats. The homogenate was administered intraperitoneally. The peritoneal cavity has been shown to present an effective surface for absorption. Specifically, Ford and Hirschman (1958) and Ford *et al* (1961) showed a rapid uptake, after intraperitoneal

injection, of amino acid into the nuclear and cortical regions of the rat brain. A significant uptake occurred within the first 4 hours after injection, with peak concentrations in the brain being reached by 24 hours. An alternative route for effective absorption of amino acid was found by Clouet and Richter (1959). Radioactive methionine was incorporated into the rat brain within 1/2-3 hours following intracisternal injection.

In the present study, only the intraperitoneal route was used for nervous tissue homogenate administration. There was no discernible effect on the liver, spleen and kidneys in rats treated with the homogenate. The major effect of the injections was a marked prolongation of the period during which neurofibrils could be found in the scar at the site of spinal cord transection. Six rats regained coordinated walking ability after six to seven weeks of complete paraplegia. In other homogenate-treated rats, the failure to develop coordinated walking may have been due to several factors. Many rats died before the time that neurofibrils could be seen throughout the scar. Many other rats developed and survived urinary tract infections; growth of the neurofibrils was possibly slowed by the concurrent disease. In some rats, with a lengthy scar between the cord ends, a longer period of homogenate injection may have benefitted the regenerative process.

The question now arises whether any specific fraction

in the nervous tissue homogenate was responsible for the beneficial effect on spinal axon regeneration. The existence of a growth-stimulating substance in the brain and spinal cord of newborn rats, not found in adult rats, is possible but unlikely. The spinal cord of the mammalian fetus and newborn does not regenerate when cut (Hooker and Nicholas. 1930; Gerard and Grinker. 1931; Hess. 1956). If a specific nerve growth-stimulating substance were present in the young mammal, the phenomenon of fetal and newborn cord regeneration might be anticipated; this has not thus far been observed. A nucleoprotein fraction of the homogenate may be postulated to have nerve stimulating properties. However, although the isolation and injection of a minute fraction of the nervous tissue homogenate might enhance the growth of peripheral axons in vivo and nerve cells in vitro, similar effects should not be anticipated in the paraplegic mammal. The catabolism, weight loss and general metabolic upheaval that accompanies the paraplegic state would seem to necessitate the concomitant administration of both a "growth stimulator" as well as the necessary materials needed for cytoplasmic synthesis and neurofibrillar growth. The administration of a total tissue homogenate of central nervous origin is most probably the method of choice in the production of central axonal regeneration.

There may be value in homogenate treatment combined with therapy to reduce scarring at the transection site. The

results of combined Piromen-homogenate therapy were, in the present study, no better than with homogenate alone. However, there appears to be merit in steroid treatment to reduce collagen fiber formation. Steroids should not be administered in doses that compound the catabolic state of the paraplegia itself. A moderate dosage of steroid has the advantage of leaving a collagenous "latticework" upon which neurofibrils are supported in their growth, as well as preventing the late formation of a dense, constricting scar at the transection site. In the present study, combined treatment with methyl prednisolone and homogenate in the early postoperative period was uniformly fatal. A depressed adrenal response to the stress of foreign protein injection has been postulated for the lethal results of the combined treatment. Nevertheless, a reduction in mesodermal scarring, by whatever means, should allow the rapidly-growing neurofibrils to pass in as direct as possible a course across the transection site. The time during which nervous tissue homogenate is required for growth might thereby be shortened.

The results of the present study have indicated that "abortive regeneration" in the spinal cord is not due primarily to scarring but to lack of a readily available reserve of the metabolites required by central neurons to meet the demands of increased axoplasmic synthesis after spinal cord transection. The fact that peripheral axons regenerate without specific chemotherapy or organotherapy may be due to differences in cellular chemistry between neurons in the central and peripheral nervous systems.

CHAPTER V

C O N C L U S I O N S

The sequelae of complete spinal cord transection have been studied in the Sprague-Dawley rat. The effects of chemotherapy and organotherapy on the process of spinal axon regeneration have been investigated. On the basis of functional, electrophysiological and histological data, the following conclusions are warranted:

1) Spontaneous regeneration of spinal axons begins in the first week after complete spinal cord transection.
2) Axonal regeneration ceases in the spinal cord of the untreated rat.
3) No functional recovery occurs in the untreated rat beyond that of the typical spinal animal.
4) Reduction of collagen fiber formation at the site of spinal cord transection following methyl prednisolone administration does not either prolong the period of spinal axon regeneration or result in functional return.
5) Bacterial pyrogen has no demonstrable effect either on scar tissue at the transection site or on functional return in the paraplegic rat.
6) The administration of a homogenate of central nervous tissue derived from newborn rats prolongs the period of spinal axon regeneration in the adult rat after complete spinal cord transection.
7) The return of motor function in the hind limbs of 6 rats treated with nervous tissue homogenate was the result of regeneration of axons in the spinal cord.
8) Regeneration of axons in the spinal cord of the rat is not inevitably an abortive process.

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